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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/40, 7 /01, C07K 14 /08, A61K 39 /12 | A2

(11) International Publication Number: **WO 00/00616**

(43) International Publication Date: 6 January 2000 (06.01.00)

(21) International Application Number: PCT/US99/14174

(22) International Filing Date: 21 June 1999 (21.06.99)

(30) Priority Data:
60/091,403 29 June 1998 (29.06.98) US

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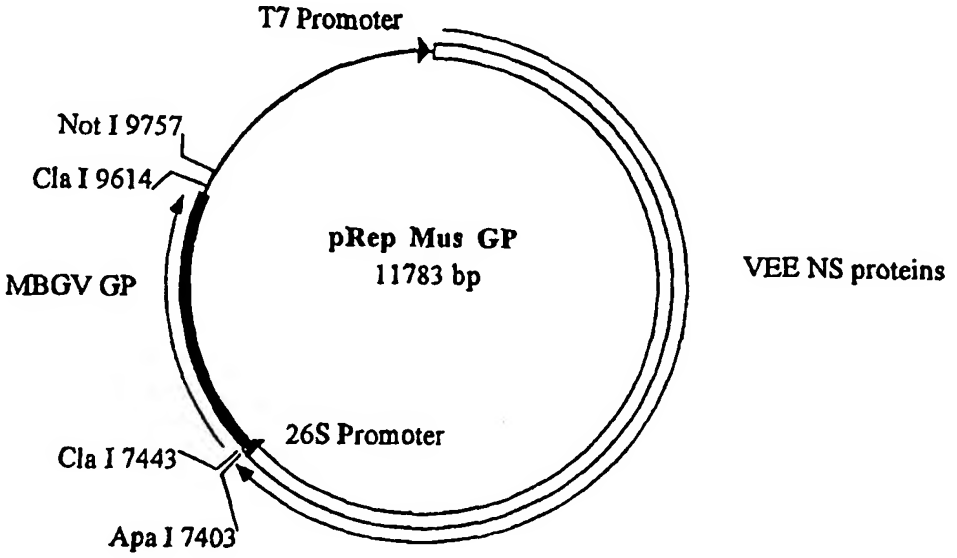
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: MARBURG VIRUS VACCINES



Map of MBGV GP replicon plasmid.

(57) Abstract

Using the MBGV GP, NP and virion proteins, a method and composition for use in inducing an immune response which is protective against infection with MBGV in nonhuman primates is described.

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TITLE OF THE INVENTION

Marburg Virus Vaccines

INTRODUCTION

Marburg virus (MBGV) was first recognized in 1967, when an outbreak of hemorrhagic fever in humans occurred in Germany and Yugoslavia, after the importation of infected monkeys from Uganda (Martini and Siegert, 1971, Marburg Virus Disease. Berlin: Springer-Verlag; Smith *et al.*, 1982, *Lancet* 1, 816-820). Thirty-one cases of MBGV hemorrhagic fever were identified that resulted in seven deaths. The filamentous morphology of the virus was later recognized to be characteristic, not only of additional MBGV isolates, but also of Ebola virus (EBOV) (Johnson *et al.*, 1977, *J. Virol.* 71, 3031-3038; Smith *et al.*, 1982, *Lancet* 1, 816-820; Pattyn *et al.*, 1977, *Lancet* 1, 573-574). MBGV and EBOV are now known to be distinctly different lineages in the family *Filoviridae*, within the viral order Mononegavirales (Kiley *et al.*, 1982, *Intervirology* 18, 24-32; Feldmann and Klenk, 1996, *Adv. Virus Res.* 47, 1-52).

Few natural outbreaks of MBGV disease have been recognized, and all proved self-limiting, with no more than two cycles of human-to-human transmission. However, the actual risks posed by MBGV to global health cannot be assessed because factors which restrict the virus to its unidentified ecological

niche in eastern Africa, and those that limit its transmissibility, remain unknown (Feldmann and Klenk, 1996, supra). Concern about MBGV is further heightened by its known stability and infectivity in aerosol form (Belanov *et al.*, 1996, *Vopr. Virusol.* 41, 32-34; Frolov and Gusev Iu, 1996, *Vopr. Virusol.* 41, 275-277). Thus, laboratory research on MBGV is necessarily performed at the highest level of biocontainment. To minimize future risk, our primary interest has been the identification of appropriate antigens and vaccine strategies that can provide immunity to MBGV.

Early efforts to demonstrate the feasibility of vaccination against MBGV were only partially successful, as inoculation with formalin-inactivated viruses only protected about half the experimental animals (guinea pigs or nonhuman primates) from fatal disease (Ignat'ev *et al.*, 1991, *Vopr. Virusol.* 36, 421-423; Ignat'ev et al., 1996, *J. Biotechnol.* 44, 111-118). We recently demonstrated that the MBGV GP, cloned into a baculovirus vector and expressed as a soluble antigen to be administered in adjuvant, was sufficient to protect most but not all guinea pigs from lethal MBGV challenge (Hevey *et al.*, 1997, *Virology* 239, 206-216). In addition, purified, ^{60}Co-irradiated virus, administered in adjuvant, completely protected guinea pigs from challenge with either of two different strains of MBGV, thus setting a standard for future, more pragmatic, vaccine candidates (Hevey *et al.*, 1997, supra). Experiences with EBOV vaccines have been similar to those with MBGV, reinforcing the difficulties of classical approaches (Lupton *et al.*, 1980, *Lancet* 2, 1294-1295). Recent efforts to develop EBOV vaccines, using three distinctly different approaches (vaccinia recombinants, VEE replicon, and

naked DNA) to achieve viral antigen expression in cells of vaccinated animals, showed that nucleoprotein (NP) as well as GP protected BALB/c mice (VanderZanden *et al.*, 1998, *Virology* 245), whereas protection of guinea pigs by NP was unsuccessful (Gilligan *et al.*, 1997, In: Brown, F., Burton, D., Doherty, P., Mekalanos, J., Norrby, E. (eds). 1997. Vaccines 97 Cold Spring Harbor Press. Cold Spring Harbor, NY; Pushko *et al.*, 1997, In: Brown, F., Burton, D., Doherty, P., Mekalanos, J., Norrby, E. (eds). 1997. Vaccines 97 Cold Spring Harbor Press. Cold Spring Harbor, NY) or equivocal (Xu *et al.*, 1998, Nat. Med. 4, 37-42).

Irrespective of how encouraging filovirus vaccine results may appear in guinea pigs or mice, protection of nonhuman primates is widely taken as the more definitive test of vaccine potential for humans. Low-passage viral isolates from fatal human cases of MBGV or EBOV tend to have uniform lethality in nonhuman primates, but not in guinea pigs or mice. Small animal models with fatal disease outcomes have been achieved only with a subset of filovirus isolates and only then by multiple serial passages in the desired host (Hevey *et al.*, 1997, supra; Connolly *et al.*, 1999, *J. Infect. Dis.* 179, suppl. 1, S203 ; Xu *et al.*, 1998, supra; Bray *et al.*, 1998, *J. Infect. Dis.* 178, 661-665). While highly useful for identification and initial characterization of vaccine candidates, guinea pig and murine models remain somewhat suspect with regard to the possibility that protection in such animals is easier to achieve than in nonhuman primates and, by inference, in humans. For example, with MBGV, peak viremias and viral titers in organs are more than

100 times higher in nonhuman primates than in guinea pigs.

Therefore, there is a need for an efficacious vaccine for MBGV useful for protecting humans against Marburg hemorrhagic fever.

SUMMARY OF THE INVENTION

The present invention satisfies the need discussed above. The present invention relates to a method and composition for use in inducing an immune response which is protective against infection with MBGV.

In this study a vaccine delivery system based on a Venezuelan equine encephalitis (VEE) virus replicon was used to identify candidate protective antigens in nonhuman primates. In this vaccine strategy, a gene coding for a protein of interest is cloned in place of the VEE virus structural genes; the result is a self-replicating RNA molecule that encodes its own replicase and transcriptase functions, and in addition makes abundant quantities of mRNA encoding the foreign protein. When replicon RNA is transfected into eukaryotic cells along with two helper RNAs that express the VEE structural proteins (glycoproteins and nucleocapsid), the replicon RNA is packaged into VEE virus-like particles by the VEE virus structural proteins, which are provided in trans. Since the helper RNAs lack packaging signals neccessary for further propagation, the resulting VEE replicon particles (VRPs) which are produced are infectious for one cycle but are defective thereafter. Upon infection of an individual cell with a VRP, an abortive infection occurs in which the infected cell produces the protein of interest in abundance, is

ultimately killed by the infection, but does not produce any viral progeny (Pushko *et al.*, 1997, *Virology* 239, 389-401). The VEE replicon is described in greater detail in U.S. Patent No. 5,792,462 issued to Johnston *et al.* on August 11, 1998.

Results shown here demonstrate that the VEE replicon is a potent tool for vaccination with MBGV antigens. Guinea pigs were protected by vaccination with packaged replicons that expressed GP, or by either of two replicons which expressed internal MBGV antigens (NP and VP35). GP expressed from the VEE replicon elicited an even more robust immunity than was achieved previously with a baculovirus-produced soluble GP administered in adjuvant. When results were extended to nonhuman primates, complete protection with GP was demonstrated. The data shown here constitute the most emphatic proof to date that an efficacious vaccine for MBGV is feasible, and define candidate antigens for such a vaccine.

Therefore, it is one object of the present invention to provide a VEE virus replicon vector comprising a VEE virus replicon and a DNA fragment encoding any of the MBGV GP, NP, VP40, VP35, VP30, and VP24, and GPΔTM, a GP deletion mutant from which the C-terminal 39 amino acids encoding the transmembrane region and cytoplasmic tail of MBGV GP were removed.

It is another object of the present invention to provide a self replicating RNA comprising the VEE virus replicon and any of the MBGV GP, GPΔTM, NP, VP40, VP35, VP30, and VP24 described above.

It is another object of the present invention to provide infectious VEE virus replicon particles produced from the VEE virus replicon RNA described above.

It is further an object of the invention to provide an immunological composition for the protection of mammals against MBGV infection comprising VEE virus replicon particles containing nucleic acids encoding any of the MBGV GP, GPΔTM, NP, VP40, VP35, VP30, and VP24 or a combination of different VEE virus replicons each containing nucleic acids encoding a different MBGV protein from any of MBGV GP, GPΔTM, NP, VP40, VP35, VP30, and VP24.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. Indirect immunofluorescence of Vero cells infected with packaged VEE replicons expressing the indicated antigens.

Figure 2. Immunoprecipitation of MBGV proteins expressed from an alphavirus replicon in Vero cells using convalescent guinea pig polyclonal anti-MBGV serum. Lane 1, cell lysate from Vero cells infected with MBGV GP replicon; lane 2, cell lysate from Vero cells infected with MBGV GPΔTM replicon; lane 3, supernatant from Vero cells infected with MBGV GPΔTM replicon; lanes 4-6, cell lysate from Vero cells infected with various clones of MBGV NP replicon; lanes 7-8, cell lysate from Vero cells infected with

various clones of MBGV VP40 replicon; lane 9, sucrose gradient-purified ^{35}S-labeled MBGV, * an unidentified 46-50 KDa protein observed in virion preparations.

Figure 3. Anti-MBGV ELISA titers of cynomolgus monkeys after three inoculations with recombinant replicon 17 days before or after challenge with MBGV. Prechallenge samples were obtained 17 days before challenge, while postchallenge samples were obtained 17 days after challenge. GP, animals inoculated with VEE replicons expressing MBGV GP; NP, animals inoculated with VEE replicon expressing MBGV NP; GP+NP, animals inoculated with a mixture of VEE replicons expressing either MBGV GP or NP; Flu HA, animals inoculated with VEE replicon expressing influenza HA. Numbers inside each symbol represent the same individual in each group. Symbols filled in with cross hatch marks signify animals that died from infection.

Figure 4. Viremia level in cynomolgus monkeys inoculated with alphavirus replicons followed by challenge with MBGV (Musoke). ● Animals vaccinated with VEE replicons expressing MBGV GP; ◆ animals vaccinated with VEE replicons expressing MBGV NP; ■, animals vaccinated with a mixture of VEE replicons which expressed either MBGV GP or NP; Δ, animals vaccinated with VEE replicons expressing influenza HA. Open symbols represent animals that died. Closed symbols represent animals that lived. Dotted line notes the lower limit of detection of this plaque assay ($1.7 Log_{10}$ PFU/ml).

Figure 5. Serum AST levels in VEE replicon inoculated cynomolgus macaques after challenge with MBGV (Musoke). ● The one animal (of six) vaccinated with VEE replicons expressing MBGV GP that exhibited AST abnormality at any time point. ◆, animals vaccinated with VEE replicons expressing MBGV NP; Δ, animals vaccinated with VEE replicon expressing influenza HA. Open symbols represent animals that died. Closed symbols represent animals that lived. Dotted line demarks 88 U/L, which is the mean (38 U/L) plus three standard deviations of pre-bleed values from the 12 monkeys in this experiment.

Figure 6: Schematic of pRep Mus GP.

Figure 7: Schematic of pRep Mus GPΔTM.

Figure 8: Schematic of pRep Mus NP.

Figure 9: Schematic of pRep Mus VP40.

Figure 10: Schematic of pRep Mus VP35.

Figure 11: Schematic of pRep Mus VP30.

Figure 12: Schematic of pRep Mus VP24.

DETAILED DESCRIPTION

In the description that follows, a number of terms used in recombinant DNA, virology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Filoviruses. The filoviruses (e.g. Marburg virus, MBGV) cause acute hemorrhagic fever characterized by high mortality. Humans can contract filoviruses by infection in endemic regions, by contact with imported primates, and by performing

scientific research with the virus. However, there currently are no available vaccines or effective therapeutic treatments for filovirus infection. The virions of filoviruses contain seven proteins which include a surface glycoprotein (GP), a nucleoprotein (NP), an RNA-dependent RNA polymerase (L), and four virion structural proteins (VP24, VP30, VP35, and VP40). Little is known about the biological functions of these proteins and it is not known which antigens significantly contribute to protection and should therefore be used to induce an immune response by an eventual vaccine candidate.

Replicon. A replicon is equivalent to a full length virus from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site. Transcription of the RNA from the replicon yields an RNA capable of initiating infection of the cell identical to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA in *trans*. This is typically done with two helpers also called defective helper RNAs. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for

subgenomic mRNA transcription and the sequences for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the cell. The particles can then by inoculated into animals similar to parent virus. The replicon particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only the viral nostructural proteins and the product of the heterologous gene cloned in place to the structural proteins.

The VEE virus replicon is a genetically reorganized version of the VEE virus genome in which the structural proteins genes are replaced with a gene from an immunogen of interest, in this invention, the MBGV virion proteins. The result is a self replicating RNA (replicon) that can be packaged into infectious particles using defective helper RNAs that encode the glycoprotein and capsid proteins of the VEE virus.

Subject. Includes both human, animal, e.g., horse, donkey, pig, guinea pig, mouse, hamster, monkey, chicken, bats, birds and insects such as mosquito.

In one embodiment, the present invention relates to a recombinant DNA molecule that includes a VEE replicon and a DNA sequence encoding any of MBGV

virion proteins GP, GPΔTM, NP, VP40, VP35, VP30, VP24. The sequences encoding the Marburg proteins GP, GPΔTM, NP, VP40, VP35, VP30, VP24 corresponding to nucleotides 104-11242 of the Genbank sequence is presented in SEQ ID NO:1; the GP DNA fragment extends from nucleotide 5932 to 8033, of which nucleotides 5940-7985 encode the protein identified in SEQ ID NO:2; the GPΔTM DNA fragment, a GP deletion mutant from which the C-terminal 39 amino acids encoding the transmembrane region and cytoplasmic tail of MBGV GP were removed, extends from nucleotides 5933 to 7869, of which nucleotides 5940-7871 encode the protein; NP, identified in SEQ ID NO:3, is encoded by the DNA fragment extending from nucleotides 104 to 2195; VP40 DNA fragment extends from nucleotide 4564 to 5958, of which nucleotides 4567-5416 encode the protein identified in SEQ ID NO:4; VP35 DNA fragment extends from nucleotide 2938 to 4336, of which nucleotides 2944-3933 encode the protein identified in SEQ ID NO:5; VP30 DNA fragment extends from nucleotide 8861 to 9979, of which nucleotides 8864-9697 encode the protein identified in SEQ ID NO:6; VP24 DNA fragment extends from nucleotide 10182 to 11242, of which nucleotides 10200-10961 encode the protein identified in SEQ ID NO:7.

When the DNA sequences described above are in a replicon expression system, such as the VEE replicon described above, the proteins can be expressed *in vivo*. The DNA sequence for any of the MBGV virion proteins described above can be cloned into the multiple cloning site of a replicon such that transcription of the RNA from the replicon yields an infectious RNA containing the sequence(s) which encodes the MBGV virion protein or proteins of interest. Use of helper RNA containing sequences

necessary for encapsulation of the viral transcript will result in the production of viral particles containing replicon RNA which are able to infect a host and initiate a single round of replication resulting in the expression of the MBGV virion proteins. Such replicon constructs include, for example, VP24 cloned into a VEE replicon, pRep Mus VP24, VP30 cloned into a VEE replicon, pRep Mus VP30, VP35 cloned into a VEE replicon, pRep Mus VP35, and VP40 cloned into a VEE replicon, pRep Mus VP40, NP cloned into a VEE replicon, pRep Mus NP, GP cloned into a VEE replicon, pRep Mus GP, GPΔTM cloned into a VEE replicon, pRep Mus GPΔTM. The sequences encoding the MBGV proteins were cloned into the replicon vector by methods known in the art and described below in Materials and Methods. Schematic diagrams of the resulting constructs are shown in the Figures. The VEE constructs containing Marburg proteins can be used as a DNA vaccine, or for the production of RNA molecules as described below.

In another embodiment, the present invention relates to RNA molecules resulting from the transcription of the constructs described above. The RNA molecules can be prepared by *in vitro* transcription using methods known in the art and described in the Examples below. Alternatively, the RNA molecules can be produced by transcription of the constructs *in vivo*, and isolating the RNA. These and other methods for obtaining RNA transcripts of the constructs are known in the art. Please see Current Protocols in Molecular Biology. Frederick M. Ausubel et al. (eds.), John Wiley and Sons, Inc. The RNA molecules can be used, for example, as a direct RNA vaccine, or to transfect cells along with RNA from helper plasmids, one of which expresses VEE

glycoproteins and the other VEE capsid proteins, as described above, in order to obtain replicon particles.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to mouse and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of MBGV virion proteins, such as glutathione S-transferase. The recombinant molecule can be suitable for transfecting eukaryotic cells, for

example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as CHO cells, vero cells, and COS cells to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art.

The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein cloned into the VEE replicon, or a source of RNA transcribed from the replicon as described above, or a source of replicon particles.

In a further embodiment, the present invention relates to a method of producing the recombinant or fusion protein which includes culturing the above-described host cells, under conditions such that the DNA fragment is expressed and the recombinant or fusion protein is produced thereby. The recombinant or fusion protein can then be isolated using methodology well known in the art. The recombinant or

fusion protein can be used as a vaccine for immunity against infection with MBGV or as a diagnostic tool for detection of MBGV infection. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit MBGV virus function, such as host proteins or chemically derived agents or other proteins which may interact with the virus to inhibit its replication or survival.

In another embodiment, the present invention relates to a MBGV vaccine comprising one or more replicon particles derived from one or more replicons encoding one or more MBGV virion proteins. The present invention relates to a method for providing immunity against MBGV virus said method comprising administering one or more replicon particles containing any combination of the MBGV virion proteins to a subject such that a protective immune reaction is generated. Even though the MBGV strain Musoke was used in the examples below, it is expected that protection would be afforded using virion proteins from other MBGV strains, as well as significant cross protection between strains.

Vaccine formulations of the present invention comprise an immunogenic amount of a replicon particle, resulting from one of the replicon constructs described above, or a combination of replicon particles as a multivalent vaccine, in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered. An amount of from about 10^5 to 10^8 or more replicon particles per dose with one to three doses one month apart is suitable, depending upon the age and species of the subject being treated. Exemplary

pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the replicon particles disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, orally and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the replicon as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed.

When the replicon RNA or DNA is used as a vaccine, the replicon RNA or DNA can be administered directly using techniques such as delivery on gold beads (gene gun), delivery by liposomes, or direct injection, among other methods known to people in the art. Any one or more constructs or replicating RNA described above can be use in any combination effective to illicit an immunogenic response in a subject. Generally, the nucleic acid vaccine administered may be in an amount of about 1-5 ug of

nucleic acid per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

The following MATERIALS AND METHODS were used in the examples that follow.

Cell cultures and viruses

Vero E6 (Vero C1008, ATCC CRL 1586), Vero 76 (ATCC CRL 1587), and BHK (ATCC CCL 10) cells were grown in minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum and gentamicin (50 µg/ml). MBGV (strain Musoke) was isolated from a human case in 1980 in Kenya (Smith *et al.*, 1982, *Lancet* 1, 816-820), and a derivative of this virus (six passages in Vero 76 cells) was used to challenge the cynomolgus monkeys. The MBGV (Musoke) that was adapted for guinea pig lethality and plaque-

picked three times was described previously (Hevey *et al.*, 1997, *Virology* 239, 206-210).

Construction of recombinant VEE replicons

MBGV gene clones pGem-GP, pGem-NP, pTM1-VP40, pTM1-VP35, pTM1-VP30, and pTM1-VP24 were generously provided by Heinz Feldmann and Anthony Sanchez (Centers for Disease Control and Prevention, Atlanta, GA) (Will et al., 1993, J. Virol. 67, 1203-1210; Sanchez et al., 1992, J. Gen. Virol. 73, 347-357; Feldman et al., 1992, Virus Res. 24, 1-19). VEE replicon and shuttle vector as well as the replicons that express Lassa virus NP and Flu HA were previously described (Pushko *et al.*, 1997, *Virology* 239, 289-401). The MBGV GP gene from pGem-GP was excised with Sal I and subcloned into the Sal I site of the shuttle vector by using standard techniques (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. 2 ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor). A clone with the MBGV GP gene in the correct orientation was excised with Apa I and Not I and this fragment was cloned into the Apa I and Not I sites of the VEE replicon plasmid.

Construction of pBluescript-KS(+)-GPΔTM, a deletion mutant of MBGV from which the C-terminal 39 amino acids (transmembrane region and cytoplasmic tail) of MBGV GP were removed, was previously described (Hevey *et al.*, 1997, supra). Here, the MBGV GPΔTM gene was excised from pBluescript-KS(+) with Hind III, and the resulting fragment ligated into the Hind III site of the shuttle vector. MBGV GPΔTM gene was excised from the shuttle vector using Cla I, and the resulting fragment ligated into the VEE replicon plasmid.

The MBGV NP gene was amplified by PCR performed with 1 ng of pGem NP as template DNA, 1 µg each of

forward (5'-CCG ACC ATG GAT TTA CAC AGT TTG TTG G-3', SEQ ID NO:8) and reverse primer (5'-CTA GCC ATG GCT GGA CTA CAA GTT CAT CGC-3' SEQ ID NO:9), and AmpliTaq polymerase (GeneAmp PCR reagent kit, Perkin Elmer, Branchburg, NJ). The primers contained an NcoI recognition sequence at the 3' terminus end (5-10 inclusive for both the forward and reverse primers). The reaction conditions were: 40 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 1 min., followed by a final extension step at 72°C for 5 min. The product was cloned into the pCR™II (InVitrogen, Carlsbad, CA) vector, excized with Eco RI, then subcloned into the shuttle vector using Eco RI sites. The MBGV NP gene was excised with Cla I and ligated into the VEE replicon plasmid.

The MBGV VP40, VP35, VP30, and VP24 genes were excised from pTM1 with Bam HI and ligated into the Bam HI site of the shuttle vector. These MBGV genes were then excised from shuttle vectors using either Cla I (VP35, VP30, and VP24) or Apa I and Not I (VP40) and ligated into the VEE replicon plasmid.

Packaging of replicons into VEE-like particles and determination of replicon titer

Replicon RNAs were packaged into VRPs as described previously (Pushko *et al.*, 1997, *Virology* 239, 389-401). Briefly, BHK cells were cotransfected with RNA transcribed *in vitro* from the replicon plasmid and from two helper plasmids, one of which expressed VEE glycoproteins and the other VEE capsid protein. The cell culture supernatant was harvested approximately 30 h after transfection and the replicon particles were concentrated and partially purified by pelleting through a 20% sucrose cushion (SW28 rotor, 25,000 rpm, 4 h), after which they were resuspending in 1 ml PBS. To assay titers of packaged replicons,

Vero cells (10^5 cells per well in eight-chamber slides, Labtek slides, Nunc Inc.) were infected with serial dilutions of the replicon particles and incubated for 16-18 h at 37°C to allow for expression of the MBGV genes. After rinsing and fixating with acetone, antigen-positive cells were identified by indirect immunofluorescence assay (IFA) as described previously (Schmaljohn *et al*, 1995, *Virology* 206, 963-972). The antibodies used included MAbs specific for MBGV GP (II-7C11), NP (III-5F8), VP40 (III-1H11), VP35 (XBC04-BG06), and VP30 (III-5F11 and 5F12) (Hevey *et al.*, 1997, supra). To detect VP24 antigen, a monkey anti-MBGV serum was used, a monkey anti-Lassa serum was used to detect expression of Lassa NP in cells, and influenza HA was detected with serum from a mouse immunized with a VEE replicon expressing influenza HA (provided by Dr. Mary Kate Hart, USAMRIID).

Immunoprecipitation and gel electrophoresis of proteins expressed by VEE replicons

Expressed MBGV antigens were immunoprecipitated and analyzed by gel electrophoresis as described previously (Hevey *et al.*, 1997, supra). Briefly, Vero cells were infected (MOI ≥ 3) with VRP expressing a single MBGV antigen. Complete medium was replaced 16-18 h postinfection by methionine- and cysteine-free medium for 1 h, and monolayers were then labeled with ^{35}S-methionine and cysteine for 4 h. Convalescent guinea pig anti-MBGV (group 1, Table 5, in Hevey *et al.*, 1997, supra) was used to immunoprecipitate MBGV-specific proteins from the resulting cell lysates.

Vaccination of guinea pigs with VEE replicons expressing MBGV proteins

Inbred strain 13 guinea pigs (maintained as a colony at USAMRIID) were inoculated subcutaneously

with 10^6 focus-forming units (FFU) of VRP in a total volume of 0.5 ml administered at two dorsal sites. Guinea pigs were anesthetized, bled, and those that received two or three doses of replicon inoculated (as described for the first vaccine dose) 28 days after the primary vaccination. Guinea pigs were anesthetized and bled again 28 days later, and animals that received three doses of replicons were inoculated, as described above. Animals were anesthetized and bled 21 days later, and challenged 7 days after the last bleed with $10^{3.0}$ plaque forming units (PFU) (ca. 2000 LD_{50}) guinea pig adapted MBGV. Animals were examined daily for signs of illness. Heparinized plasma was obtained from the retroorbital sinus of anesthetized animals 7 days postinfection for assay of viremia. Surviving guinea pigs were observed for at least 30 days after challenge, then anesthetized and exsanguinated. Viremia titers was measured by plaque assay on Vero E6 cells.

Vaccination of cynomolgus monkeys with replicons

Twelve cynomolgus macaques (*Macaca fascicularis*), 11 females and 1 male, ranging from 2.8 to 4.5 Kg, were inoculated subcutaneously with 10^7 FFU of VRP in a total volume of 0.5 ml at one site. Monkeys were anesthetized with ketamine, bled, and inoculated (as described for the first vaccine dose) 28 days after the primary injection, and again 28 days after the second. Animals were anesthetized and bled 21 days after the third vaccine dose, then were challenged 14 days later with $10^{3.9}$ PFU MBGV subcutaneously. Here and in guinea pig experiments, the inoculum was back-titrated to ensure proper dose delivery. Animals were examined daily by the attending veterinarian for signs

of illness, and given buprenorphine (Buprenex) at a dosage of 0.01 mg/kg body weight, to provide analgesia upon signs of distress. Of the unprotected animals, three succumbed abruptly, while one was euthanized in extremis. A detailed clinical evaluation, serum for viremia determination and blood chemistries, as well as EDTA blood was obtained from anesthetized animals 17 days before and 3, 5, 7, 10, 17, and 32 days postinfection. Viremia was measured by plaque assay on Vero E6 cells.

MBGV ELISA and infectivity assays

Antibody titers in guinea pig plasmas or monkey sera were determined by an indirect ELISA as described previously (Hevey *et al.*, 1997, supra). Briefly, antigen consisting of purified, irradiated virus was coated directly onto PVC plates and serial dilutions of test serum were added to wells containing antigen. The presence of bound antibody was detected by use of the appropriate horseradish peroxidase conjugated anti-species antibody (HPO-goat-anti-guinea pig IgG H+L; HPO-goat-anti-monkey IgG H+L). Endpoint of reactivity was defined as the dilution at which OD_{405} was 0.2 as determined by extrapolation of a four parameter curve fit (SOFTmax®, Molecular Devices Corp. Sunnydale, CA) of background-subtracted mean OD versus dilution. Results shown in any table or figure are from a single assay to allow more valid comparison of endpoints. Plaque assays were performed on Vero E6 cells with a semi-solid overlay on serial dilutions of samples. Viral plaques were visualized by staining viable cells with neutral red 6-7 days postinfection. To measure plaque reduction neutralization, equal volumes of a virus stock (target plaque dose was 100 PFU) and serum diluted in cell culture medium were mixed and incubated at 37°C for 1 h. The resulting

sample was assayed by plaque assay on Vero E6 cells for more than a 50 % reduction in PFU compared to control samples.

Clinical laboratory assays

For nonhuman primate studies, hematological results were obtained with a Coulter instrument, and differential counts were performed manually. Clinical chemistry results were obtained with a Piccolo™ analyzer (Abaxis, Inc., Sunnydale, CA) using the diagnostic panel General Chemistry 12, which measures alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), amylase, aspartate aminotransferase (AST), calcium, cholesterol, creatinine, glucose, total bilirubin, total protein, and urea nitrogen.

Example 1

Analysis of protein products synthesized after infection of Vero cells with VEE replicons that expressed MBGV proteins

Results of indirect immunofluorescence assay (IFA) analyses of Vero cells infected with different recombinant VEE replicons expressing MBGV proteins, are shown in Figure 1. Expression of the indicated protein products was detected both with polyclonal guinea pig anti-MBGV and with monoclonal antibodies (MAbs) specific for the indicated MBGV proteins or, in the case of VP24 (for which no MAbs were available), with convalescent serum from a monkey that had survived infection with MBGV. There were distinct staining patterns for several of the expressed proteins. MBGV GP was observed as a plasma membrane fluorescence, while the GPΔTM provided a more diffuse cytoplasmic staining. These different staining patterns were not unexpected as GPΔTM, which lacks the hydrophobic transmembrane region of GP, is a secreted product. MBGV NP and VP35 formed discrete patterns in

the cytoplasm of cells. MBGV VP40 demonstrated a more diffuse cytoplasmic staining pattern. MBGV VP30 was present in unique large globules staining in the cytoplasm of cells. MBGV VP24 staining was typically perinuclear. In summary, IFA served to assure that the appropriate antigen was expressed in a given preparation; it highlighted staining patterns, which demonstrated the localization of the expressed MBGV proteins in Vero cells; and it served as the basis for the assay whereby 10-fold dilutions of VRPs were quantitated for infectivity, as focus forming units (FFU).

Expression, antigenicity, and size determination of the MBGV proteins were confirmed by immunoprecipitation and gel electrophoresis. The results obtained from expression of MBGV GP, GPΔTM, NP, and VP40 in Vero cells are shown in Figure 2. Products of the expected sizes were specifically immunoprecipitated from replicon-infected cell lysates. Glycosylation of MBGV GP more than doubles the predicted size of the peptide chain, and typically results in a heterogeneous array of posttranslationally modified products (Feldmann *et al.*, 1991, *Virology* 182, 353-356; Feldman et al., 1994, *Virology* 199, 469-473), especially in GP from cell lysates, as shown in Figure 2, lane 1. As expected and shown previously in the baculovirus system, GPΔTM was secreted, and thus present in the supernatant of replicon-infected cells (Fig. 2, Lane 3). Appropriately, both the cell-associated (lane 2) and secreted (lane 3) forms of GPΔTM appeared smaller than the largest forms of GP (lane 1). The secreted form of GPΔTM appeared larger and somewhat more homogeneous than the same molecule from cell lysates, as noted previously (Hevey *et al.*, 1997, supra)

(compare Fig. 2, Lanes 2 and 3). This difference likely reflects the more complete glycosylation of the secreted product compared to partially glycosylated forms of this protein expected to be present in the cell. In this gel, and with considerably less intensity in other preparations, an unidentified protein of approximately 46 KDa, which can be immunoprecipitated with GP-specific monoclonal antibodies (not shown), is evident in MBGV virions (Fig. 2, Lane 9). Although it remains to be confirmed, this product may be the glycosylated form of a putative 27 KDa cleavage product of GP, reported to be the result of a posttranslational, furin-mediated cleavage of GP (Volchkov *et al*, 1998, *Proc. Natl. Acad. Sci. USA* 95:5762-5767). Replicon-expressed MBGV NP (Fig. 2, Lanes 4-6) and VP40 (Fig. 2, Lanes 7-8) comigrated with the authentic proteins present in purified MBGV virions. In other experiments, the reactivity with polyclonal or MAbs and the authentic electrophoretic migrations of the remaining replicon-expressed MBGV proteins (VP30, VP35, and VP24) were similarly demonstrated (data not shown).

Example 2

Protective efficacy of VEE replicons expressing MBGV proteins in strain 13 guinea pigs

Groups of strain 13 guinea pigs were inoculated with packaged recombinant VEE replicons expressing individual MBGV proteins, and later challenged with $10^{3.3}$ LD_{50} guinea pig-adapted MBGV subcutaneously. Results are shown in Table 1. MBGV GP protected guinea pigs from both death and viremia when administered as a three dose regimen. In addition, no reduction in efficacy or potency was observed when a

two dose regimen was instituted, and significant efficacy was observed even when a single dose of 10^6 FFU of VRP expressing MBGV GP was used as an immunogen. The efficacy of either the two or three dose vaccine schedule was further demonstrated by the observation that no boost in postchallenge ELISA titers were observed. This result suggested minimal antigen exposure after challenge with MBGV, and thus robust or even sterile immunity in these animals. MBGV GPΔTM, which was previously shown to be protective as a vaccine when produced from insect cells, also protected guinea pigs from death and viremia when delivered in an VEE virus replicon. Again, there were no increases in postchallenge ELISA titers in the group of animals immunized with GPΔTM, thus no differences were discerned in the vaccine efficacy of membrane-bound versus soluble GP.

Table 1

Protection of replicon inoculated strain 13 guinea pigs from lethal challenge with Marburg virus (Musoke isolate)

		Log 10 ELISA Titer*					
	# of Doses						
Antigen	Replicon	S/T[a]	Day-7	Day 64	Viremia[b]	V/T[c]	MDD
GP	3	6/6**	4.21	3.80	<1.7	0/6	-
GP	2	6/7**	4.30	4.06	<1.7	0/6	-
GP	1	5/6*	2.89	4.19	4.1	1/6	9
NP	3	6/6**	3.38	3.94	<1.7	0/6	-
VP40	3	1/6	2.83	2.68	4.5	5/6	10
GPΔTM	3	6/6**	3.93	3.65	<1.7	0/6	-
VP35	3	5/6*	1.99	3.75	3.7	5/6	13
VP30	3	0/6	2.23	-	5.8	6/6	10
VP24	3	1/6	<1.5	4.31	5.6	6/6	11
Lassa NP	3	1/6	<1.5	4.19	6.0	5/6	10
None	-	1/6	<1.5	4.25	5.2	5/6	11

*Endpoint titer of equal volumes of serum pooled from animals in each group against MBGV Musoke

[a] Survivors/Total (S/T) on day 30 postinfection. ** indicates $p<0.01$, * indicates $p<0.05$.

[b] Viremia (Log_{10} PFU/ml) day 7 postinfection. Where ≥2 animals were viremic, a GMT was calculated.

[c] Viremic animals/total (V/T) on day 7 postinfection. All animals that died were viremic.

In the experiment shown, MBGV NP protected all vaccinated guinea pigs from both viremia and death, while MBGV VP35 vaccination resulted in five of six animals surviving, but four of the five survivors were viremic seven days postinfection. None of the other MBGV viral proteins cloned into VEE replicons evoked significant protection against a lethal challenge with MBGV. Thus, the proteins that showed the most promise as vaccine candidates in the guinea pig model were MBGV GP and NP. Cumulative results from this and additional experiments (not shown) in strain 13 guinea pigs inoculated three times with VRPs demonstrated complete survival with GP (18/18), and less complete protection with NP (16/18) and VP35 (13/18) as compared with controls (2/24).

Example 3

Protection of cynomolgus monkeys vaccinated with recombinant VEE replicons expressing either MBGV GP and/or NP

Encouraged by the success in vaccinating guinea pigs against MBGV, we evaluated the ability of these same VEE replicons to protect cynomolgus macaques from lethal MBGV infection. The monkeys received 10-fold higher doses of replicons, but on an identical schedule as tested in the guinea pigs. Four groups contained three monkeys each. One group received VRPs which expressed MBGV GP; a second group received VRPs

which expressed MBGV NP; a third group received a mixture of MBGV GP and MBGV NP VRPs; and a fourth received VRPs which expressed a control antigen (influenza HA) irrelevant to MBGV immunity. Anti-MBGV ELISA antibody titers were monitored throughout the experiment.

All animals that received VEE replicons expressing MBGV GP, either alone or in combination with MBGV NP, survived challenge with 8000 PFU MBGV without any observed signs of illness (Table 2). Of the three animals vaccinated with MBGV NP, one died 8 days after challenge from MBGV disease. The other two NP recipients displayed signs of illness 7-9 days after challenge, but eventually recovered. One NP-inoculated survivor had a relatively mild disease (slightly reduced activity and responsiveness), while the other had severe disease which included obvious petechiae, loss of weight, reduced activity, and fever. All control animals succumbed, with clinical signs first noted on day 7 or 8, and deaths occurring on days 9 or 10 postchallenge.

Table 2

Survival of replicon-inoculated cynomolgus monkeys•

Replicon[a]	Survival/Total	Sick/Total	Day of Death
GP	3/3*	0/3	-
NP	2/3	3/3	8
GP + NP	3/3*	0/3	-
Influenza HA	0/3	3/3	9,9,10

• surviving animals remain healty >90 days postchallenge.
[a] Antigen delivered by VEE replicon.
* Indicates p=0.05.

The pre- and postchallenge ELISA antibody titers of the cynomolgus macaques are shown in Figure 3. All

animals inoculated with replicons that expressed MBGV proteins demonstrated prechallenge ELISA titers to purified MBGV antigen. Of the three GP-vaccinated animals that survived challenge, two demonstrated a modest boost in ELISA antibody titer (10-30 fold) when pre- and postchallenge samples were compared. The two surviving NP-inoculated macaques had larger boosts in ELISA antibody titers (100-300 fold) when pre- and postchallenge samples were compared. Two of three animals vaccinated with both GP and NP also demonstrated 100- to 300-fold rise in ELISA titers. These observations, in conjuction with the back titration of the MBGV challenge inoculum (8000 PFU), confirmed that all groups were unambiguously challenged, and that two monkeys had particularly robust immunity that apparently restricted virus replication below an immunogenic threshold.

A plaque reduction neutralization assay was performed on pre- and postchallenge serum samples. No neutralization activity was observed, at 1:20 or higher dilutions, in any sample. It should be noted that it is frequently difficult to demonstrate filovirus neutralizing antibody *in vitro*; however, antibodies may nonetheless be relevant *in vivo* (Hevey *et al.*, 1997, *Virology* 239, 206-216), perhaps via mechanisms other than classical neutralization (Schmaljohn *et al.*, 1982, supra).

The viremia levels in each of the monkeys at several time points after MBGV challenge are shown in Figure 4. The data illustrate the profound differences between lethally infected control animals and healthy survivors. Most striking, none of the animals vaccinated with GP, either alone or in combination with NP, had infectious MBGV virus in their sera that was detectable by plaque assay.

Animals vaccinated with a replicon expressing influenza HA were all viremic by day 3 postchallenge and demonstrated sharp rises in MBGV viremia levels which peaked at 7.5-8.0 Log_{10} PFU/ml on day 7 postinfection. Among monkeys vaccinated with NP, one died with viremias indistinguishable from controls. In contrast, the two NP-vaccinated monkeys that recovered had peak viremias that were diminished ≥1000 fold compared with controls. By day 10 postinfection, the NP-vaccinated monkey with the milder illness had no detectable viremia, while the more severely affected monkey still had ~ 4.5 Log_{10} PFU/ml virus. By day 17 postinfection no viremia was detectable in either of the surviving NP vaccinated animals.

Example 4

Additional measures of vaccine-mediated protection

Upon necropsy of the control and the unprotected NP-inoculated monkeys, MBGV titers in their livers were 9.2, 9.7, 9.4, and 9.6 Log_{10} PFU/gm. Virus was detected in all other organs examined as well, and although abundant, was at least 10-fold lower than in the liver. Not surprisingly, elevated liver enzymes were the most obvious abnormal feature in clinical chemistries. As shown in Figure 5, unprotected monkeys had elevated AST levels by day 5 or 7 postinfection, and these were paralleled by similarly profound increases in ALT and ALP (not shown). Terminal samples were automatically rejected by the instrument as too lipemic or hemolyzed; however, in a previous set of control monkeys liver enzymes had continued to ascend dramatically (not shown). With regard to vaccine-mediated protection, it is instructive that the two NP-inoculated survivors

exhibited marked but transient rises in their liver enzymes (Fig. 5), which is consistent with their viremias and signs of MBGV disease. Also, the more severely affected NP- inoculated survivor exhibited a transient rise in urea nitrogen and creatinine (not shown), coincident with recovery and viral clearance. This may have been due to virus-antibody complexes perturbing kidney function, or to direct viral damage to the organs. In contrast, the six monkeys vaccinated with GP exhibited either a minimal rise at one time point (i.e., the one GP animal shown in Fig. 5) or no significant increases in liver enzymes at any time evaluated. Other clinical chemistries and hematological findings remained normal in MBGV-inoculated macaques vaccinated previously with GP or GP+NP, in contrast with control monkeys that exhibited the expected profound end-stage abnormalities in both hematological and chemistry measurements (Johnson *et al.*, 1995, *Int. J. Exp. Pathol.* 76, 227-236).

Discussion

To our knowledge, this is the first report of any filovirus vaccine shown to be completely efficacious in nonhuman primates. Before these observations, we were cautiously optimistic about the overall feasibility of an efficacious vaccine for MBGV, but were also concerned that proofs of filovirus vaccine concepts in guinea pigs may not necessarily forecast success in nonhuman primates and, by inference, in humans. Results presented here defined GP, possibly in combination with NP, as candidate antigens for a MBGV vaccine, and demonstrated that nearly complete immunity is achievable in nonhuman primates.

We chose an alphavirus replicon based on VEE virus to deliver the antigens of interest. This method of vaccination has several advantages (Pushko

et al., 1997, *Virology* 239, 389-401), including the ability to produce large quantities of antigen *in situ*, so that native processing of the antigens might evoke a broad array of immune responses. In addition, all transcription of RNA occurs in the cytoplasm of cells, which avoids RNA splicing problems sometimes observed when proteins of RNA viruses are expressed from the nucleus. Moreover, VEE replicons have proven stable after packaging into VRPs. In addition to robust antibody induction, alphavirus replicons have been demonstrated to elicit cytotoxic T lymphocytes in mice (Caley *et al.*, 1997, *J. Virol.* 71, 3031-3038; Zhou *et al.*, 1995, *Proc. Natl. Acad. Sci. U.S.A.* 92, 3009-3013). The success reported here using VEE replicons to vaccinate monkeys against lethal MBGV challenge justifies a more detailed analysis of the potential of these vectors for use as human vaccines. These analyses may include such factors as the relevance of host-vector interactions that may affect vaccine potency, overall safety of the vector, and the duration and minimal requirements for immunity to MBGV disease induced by this vector.

Two viral antigens demonstrated unambiguous potential as protective antigens in the guinea pig model: MBGV GP and MBGV NP. Another viral antigen, VP35, provided significant protection from death; however, most (5/6) animals vaccinated with VP35 exhibited viremias 7 days after infection. Consequently, VP35 was not considered a candidate for the initial examination of vaccine efficacy in nonhuman primates. While none of the other viral antigens showed significant promise as protective antigens in the guinea pig model, some were only weakly immunogenic, at least when delivered as VRPs. Thus, we have not formally excluded the possibility

that such antigens may prove protective under different circumstances, or in species other than guinea pigs.

As a more definitive test of efficacy, the two most promising guinea pig protective antigens from MBGV were used to inoculate nonhuman primates either alone or in combination. Using recombinant VEE replicons, MBGV GP was clearly shown to be protective. The observation that none of the animals developed overt illness or viremia was conclusive proof that this vaccine approach had protected animals from a substantial challenge dose of MBGV. However, there were some significant differences observed between guinea pigs and cynomolgus macaques. Most notable was the observation that two-thirds of the GP-vaccinated monkeys demonstrated rises in ELISA antibody titers following MBGV challenge, whereas there was apparently sterile immunity (i.e. no further increases in antibody titers) to viral challenge in guinea pigs given a 10-fold lower dose of the same vaccine. This may be attributable to the overall higher prechallenge ELISA antibody titers observed in guinea pigs when compared to those observed in the monkeys (Table 1 vs. Fig. 3).

The second antigen examined, MBGV NP, was less effective at protecting nonhuman primates compared to guinea pigs. All the monkeys inoculated with NP displayed signs of illness, with one animal dying in the same time frame as control animals. All animals were viremic, and viremia levels were predictive of outcome. As expected, the two animals that survived illness had large boosts in their ELISA antibody titers against MBGV when pre- and postchallenge sera were examined. Though not statistically significant in a group of only three animals, MBGV NP was

apparently able to provide a measure of protection from death, but not from disease in two monkeys. We surmise that the immune response to NP was sufficient to suppress replication of MBGV until augmented by additional host immune responses.

The monkeys that were vaccinated with both MBGV GP and NP demonstrated the same degree of protection as the animals vaccinated with GP alone. No viremias were observed at any time point, and two of three animals demonstrated postchallenge increases in ELISA antibody titers to MBGV. These results demonstrated that the NP replicon, equivocal by itself as a macaque vaccine, did not interfere with a GP-based vaccine when protective efficacy was used as a measurement.

For these studies, in the interest of expedient vaccine development, protection from viral disease was prioritized over the detailed study of immune mechanisms in two relatively difficult animal species for immunological studies, guinea pigs and cynomolgus macaques. It was already clear from studies done in guinea pigs that ELISA antibody titers to MBGV were not wholly predictive of clinical outcome, but rather one measure of immunogenicity of the vaccine candidate. However, it was also known that administration of polyclonal antisera or a neutralizing MAb could protect some guinea pigs from lethal challenge, indicating that antibodies can play a role in the protective response to MBGV (Hevey *et al.*, 1997, supra). As for immunity to virtually all viruses, T cell responses to MBGV are almost certainly important in their immunoregulatory and effector functions. Indeed, we observed protection in both guinea pigs (NP and VP35) and nonhuman primates (NP) with antigens for which the most logical protective mechanisms involve cellular immunity. However, it

also proved emphatically true in the most susceptible animals -- nonhuman primates -- that protective immunity was elicited by an antigen (GP) that theoretically favored a redundant protective response of both T cells and antibodies.

What is claimed is:

1. A recombinant DNA construct comprising:

(i) a vector, and

(ii) at least one of the MBGV virus DNA fragments encoding any one of GP, NP, VP40, VP35, VP30, VP24, and GPΔTM.

2. A recombinant DNA construct according to claim 1 wherein said vector is an expression vector.

3. A recombinant DNA construct according to claim 1 wherein said vector is a prokaryotic vector.

4. A recombinant DNA construct according to claim 1 wherein said vector is a eukaryotic vector.

5. The recombinant DNA construct of claim 1 wherein said vector is a VEE virus replicon vector.

6. The recombinant DNA construct according to claim 5 wherein said MBGV virus proteins are from strain Musoke.

7. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus GP.

8. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus NP.

9. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus VP40.

10. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus VP35.

11. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus VP30.

12. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus VP24.

13. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus GPΔTM.

14. Self replicating RNA produced from the construct of any of claim 7, 8, 9, 10, 11, 12, or 13.

15. Infectious alphavirus particles produced from packaging the self replicating RNA of claim 14.

16. A pharmaceutical composition comprising infectious alphavirus particles according to claim 15 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

17. A host cell transformed with a recombinant DNA construct according to claim 5.

18. A host cell according to claim 17 wherein said host cell is prokaryotic.

19. A host cell according to claim 17 wherein said host cell is eukaryotic.

20. A method for producing MBGV virus proteins comprising culturing the cells according to claim 18 under conditions such that said DNA fragment is expressed and said MBGV protein is produced.

21. A method for producing MBGV proteins comprising culturing the cells according to claim 19 under conditions such that said DNA fragment is expressed and said MBGV protein is produced.

22. A vaccine for MBGV comprising viral particles containing one or more replicon RNA encoding one or more MBGV proteins selected from the group consisting of GP, NP, VP24, VP30, VP35, VP40, and GPΔTM.

23. A pharmaceutical composition comprising the self replication RNA of claim 14 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

24. A pharmaceutical composition comprising one or more recombinant DNA constructs chosen from the group consisting of pRep Mus GP, pRep Mus GPΔTM, pRep Mus NP, pRep Mus VP40, pRep Mus VP35, pRep Mus VP30, pRep Mus VP24 in a pharmaceutically acceptable amount, in a pharmaceutically accpetable carrier/and or adjuvant.

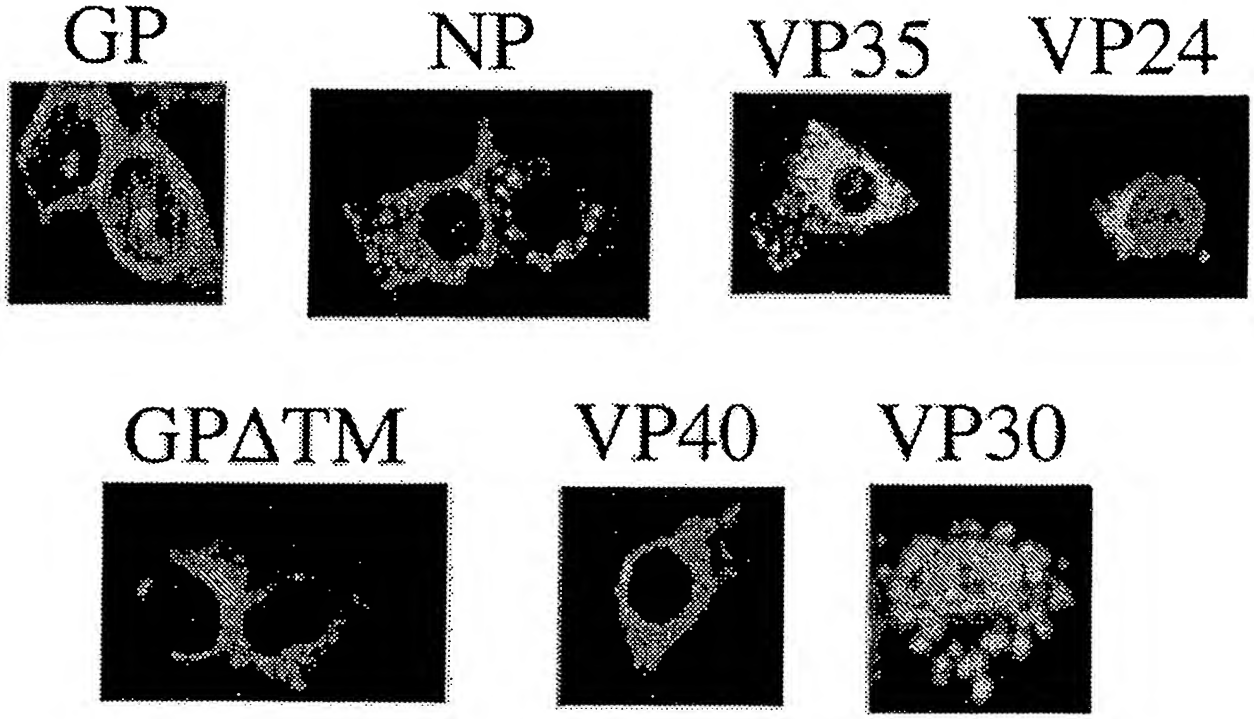


Fig 1

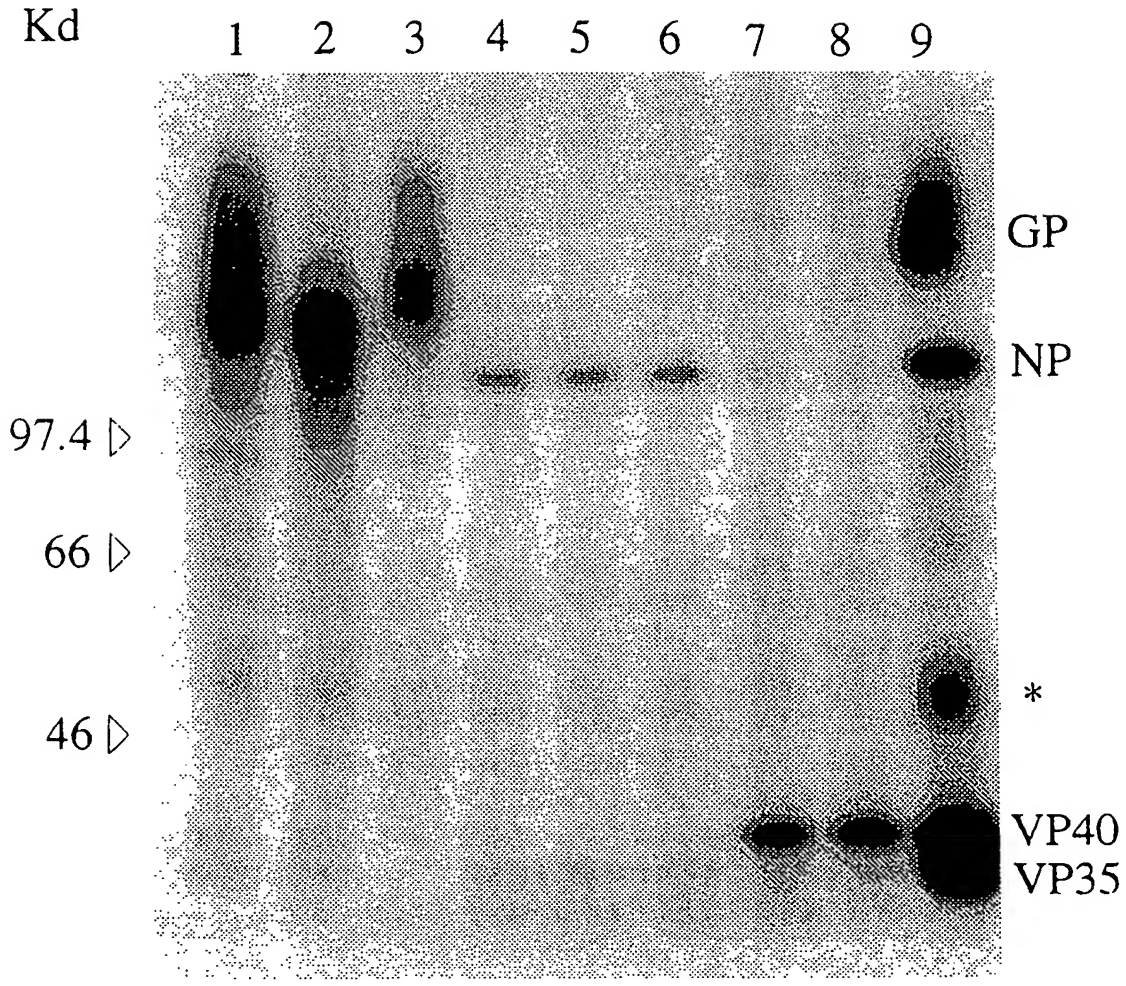
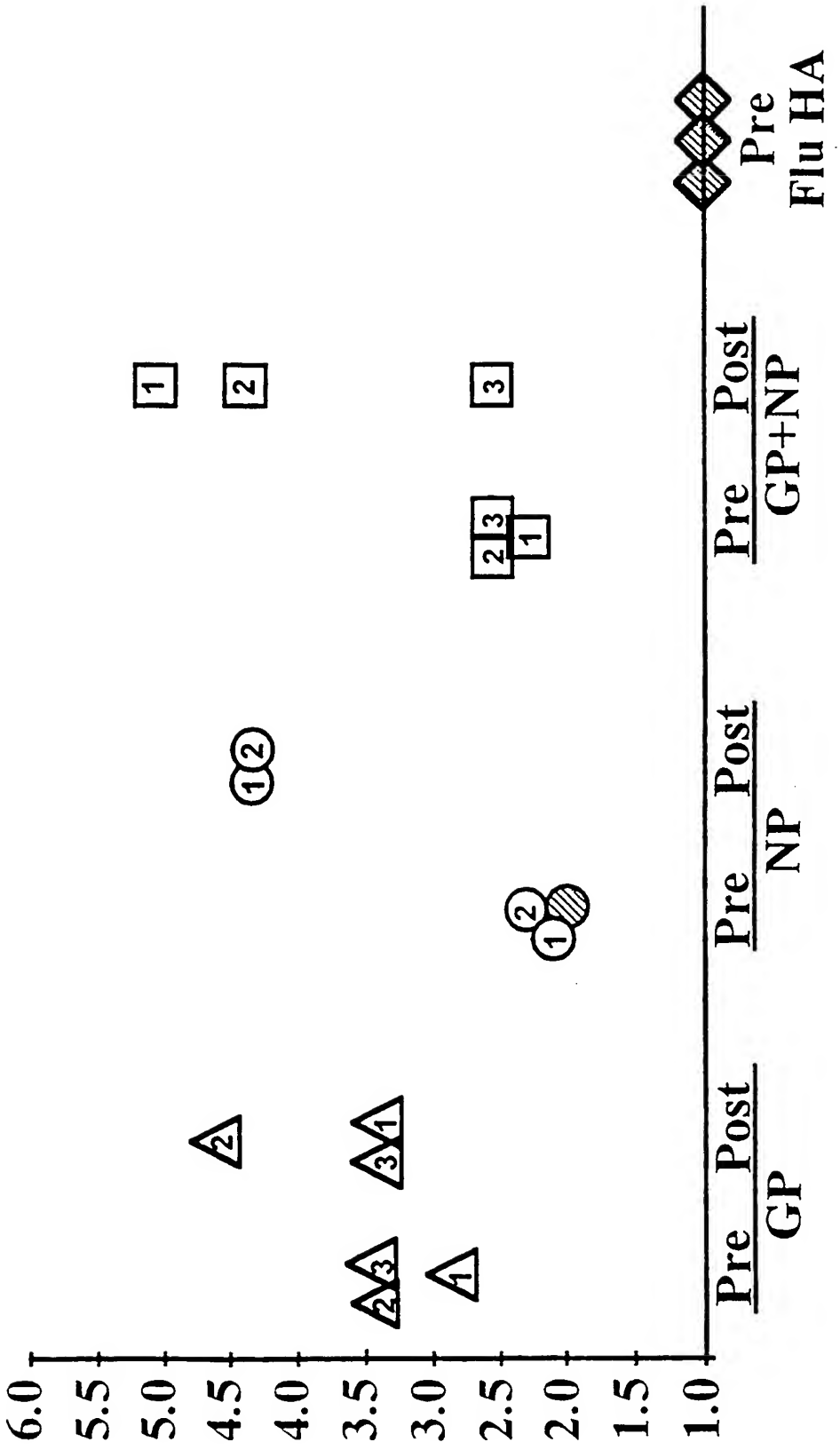


Fig 2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5.5
5.0
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3.0
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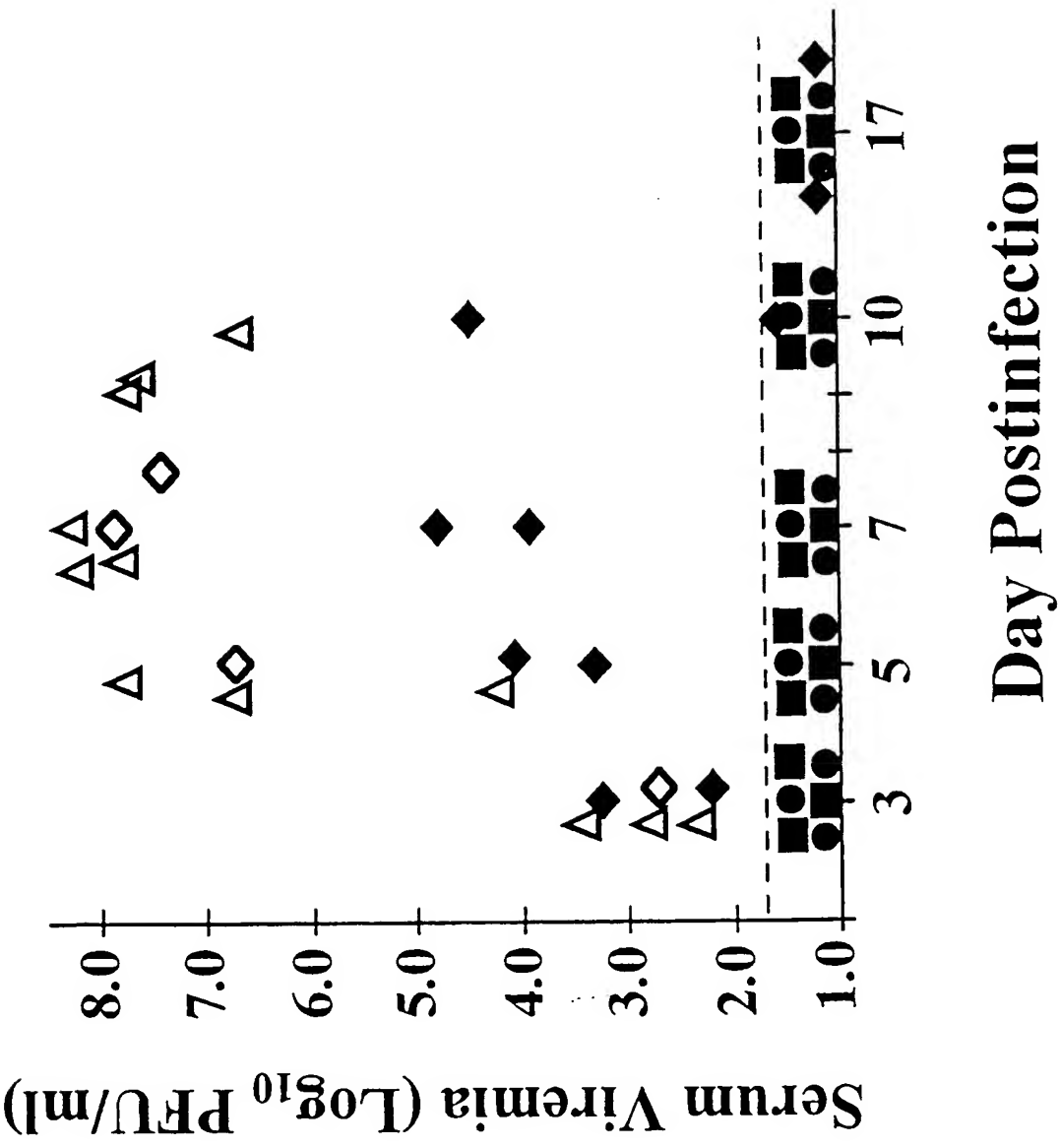
Pre Post
GP
Pre Post
NP
Pre Post
GP+NP
Pre
Flu HA
Replicon-Delivered Antigen

Fig 3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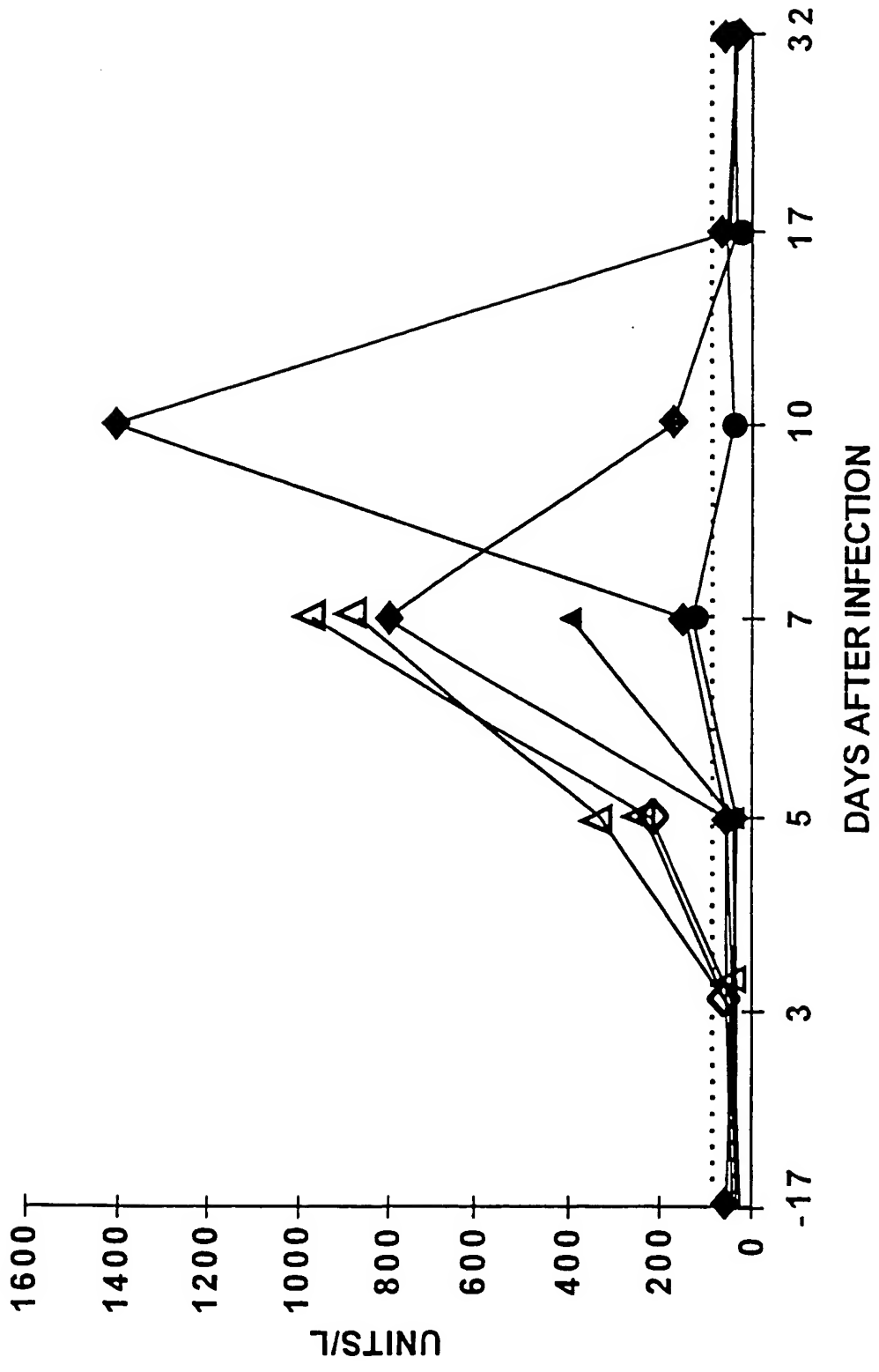
7.0
6.0
5.0
4.0
3.0
2.0
1.0
3
5
7
10
17
Day Postinfection
GP
NP
GP+NP
HA

Fig 4

Fig. 5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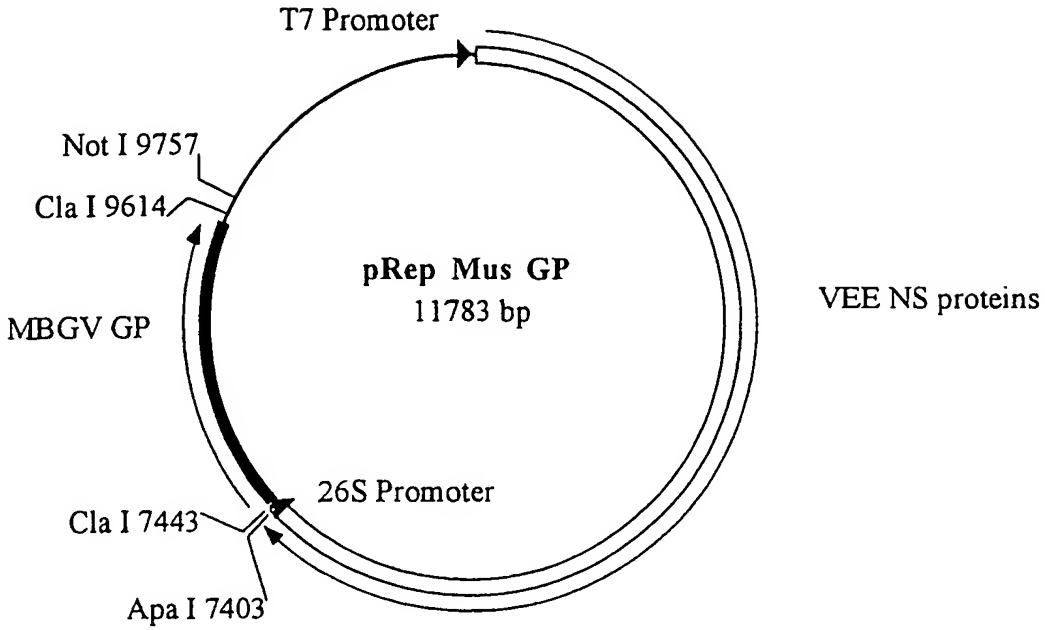

Figure 6: Map of MBGV GP replicon plasmid.

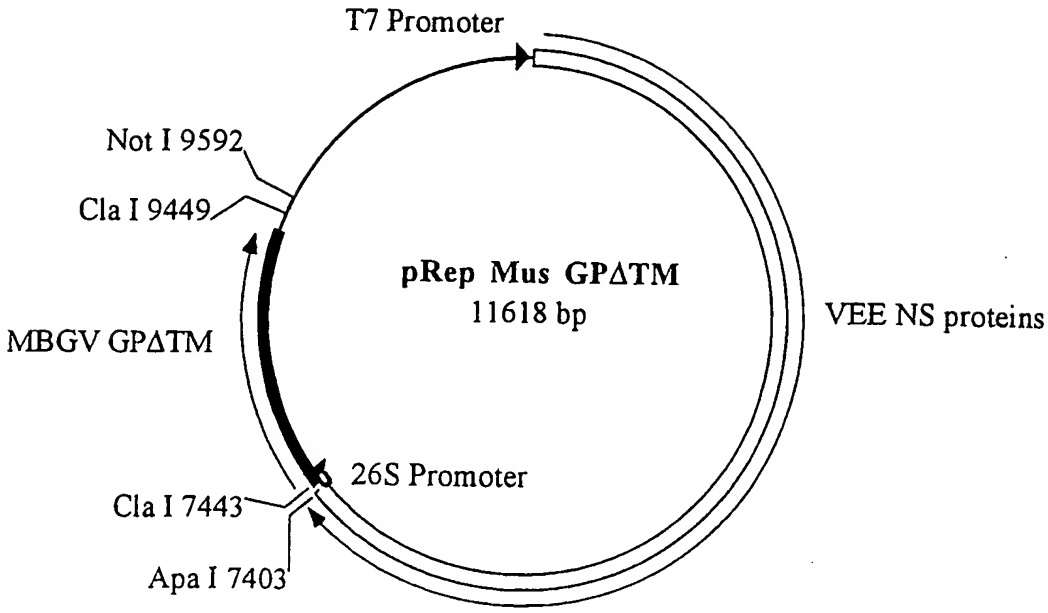

Figure 7: Map of MBGV GPΔTM replicon plasmid

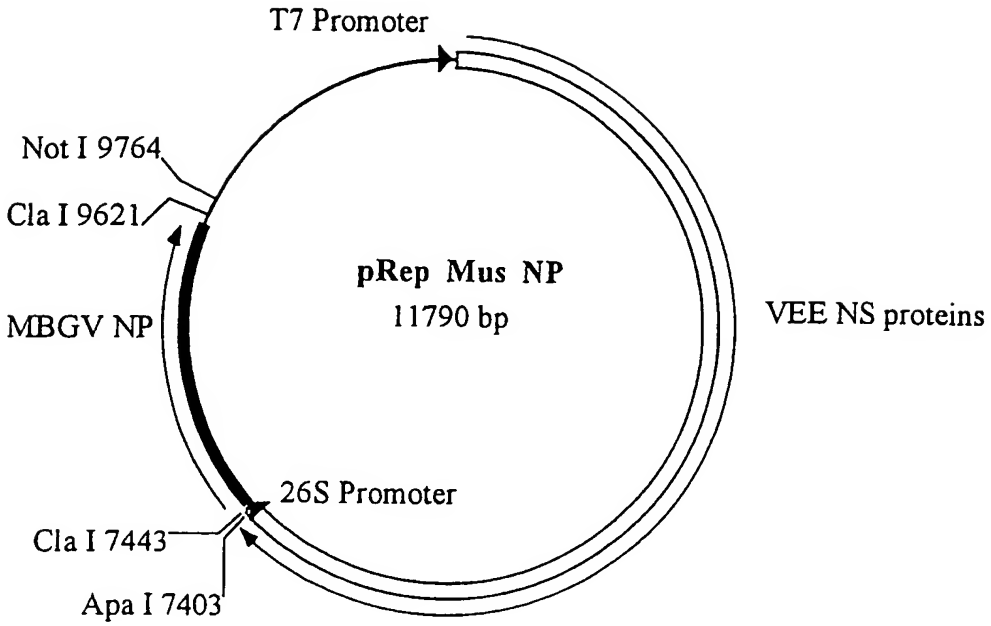

Figure 8: Map of MBGV NP replicon plasmid.

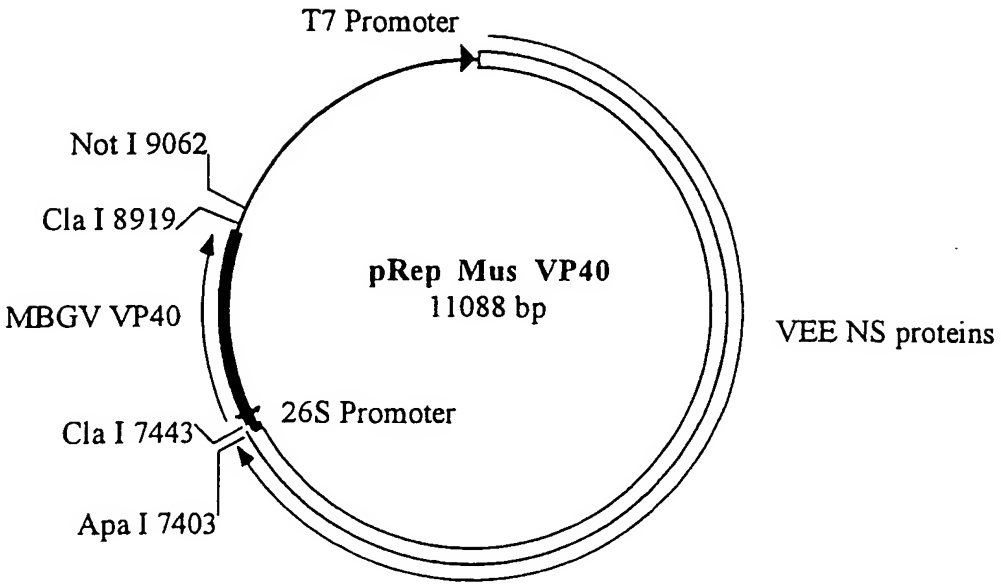

Figure 9: Map of MBGV VP40 replicon plasmid.

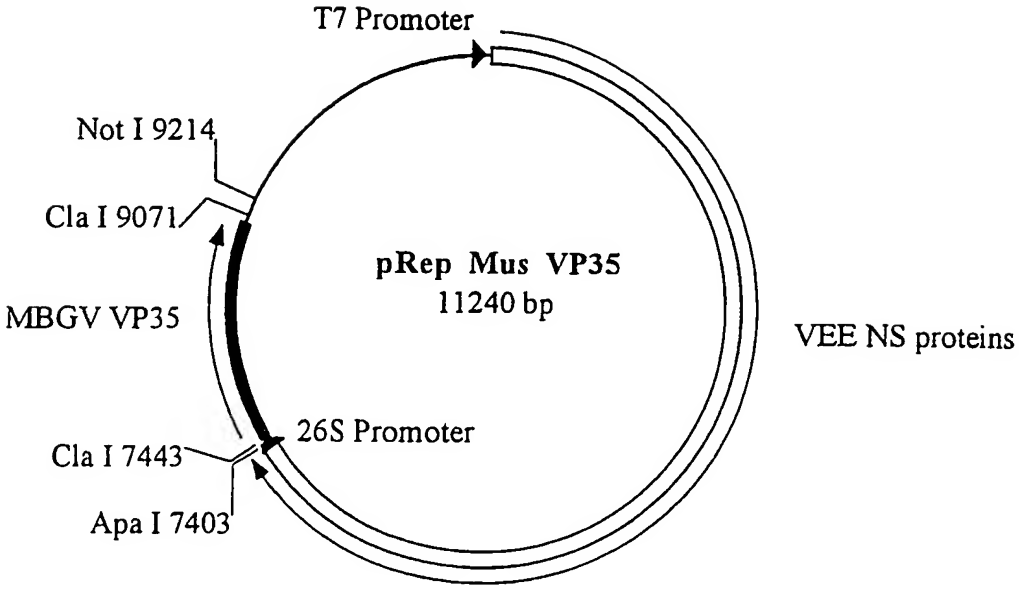

Figure 10: Map of MBGV VP35 replicon plasmid.

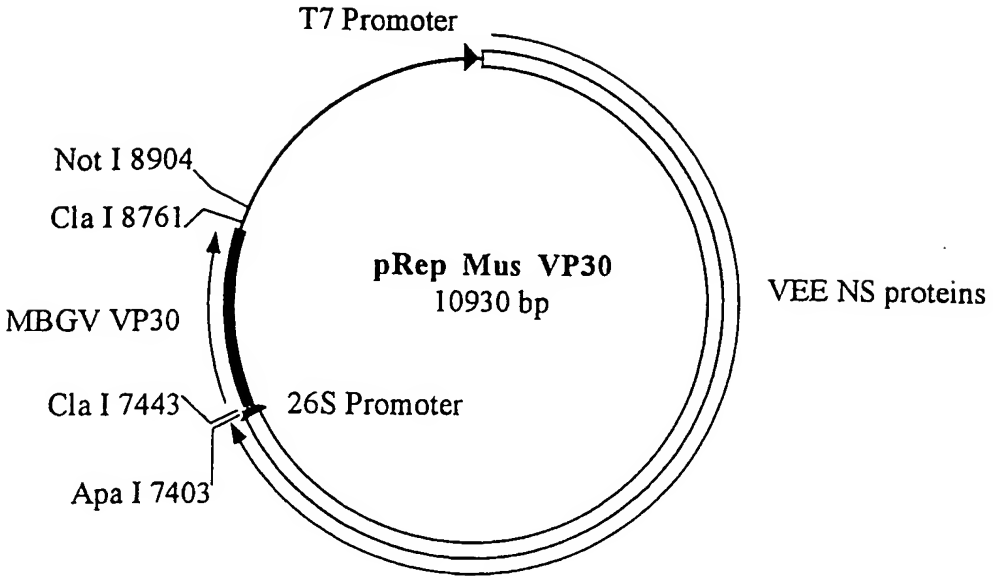

Figure 11: Map of MBGV VP30 replicon plasmid

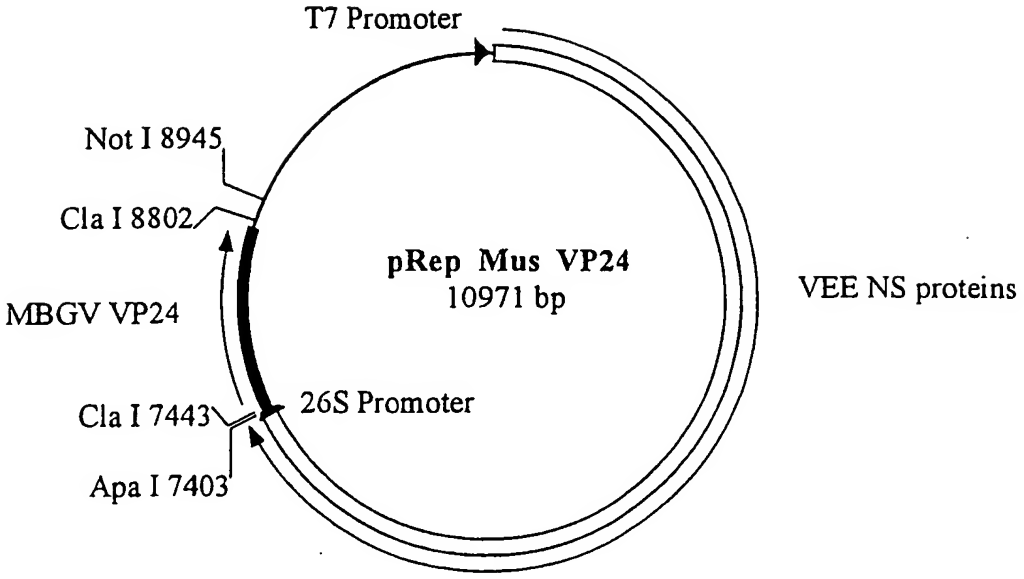

Figure 12: Map of MBGV VP24 replicon plasmid.

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ACCESSION Z12132 S55429
NID g541780
VERSION Z12132.1 GI:541780
KEYWORDS glycoprotein; nucleoprotein; polymerase; vp24 protein; vp30 protein; vp35 protein; vp40 protein.
SOURCE Marburg virus.
ORGANISM Marburg virus
Viruses; ssRNA negative-strand viruses; Mononegavirales;
Filoviridae; Filovirus.
REFERENCE 1 (bases 1 to 19104)
AUTHORS Feldmann,H., Muhlberger,E., Randolf,A., Will,C., Kiley,M.P., Sanchez,A. and Klenk,H.D.
TITLE Marburg virus, a filovirus: messenger RNAs, gene order, and regulatory elements of the replication cycle
JOURNAL Virus Res. 24 (1), 1-19 (1992)
MEDLINE 92327834
REMARK (sites)
REFERENCE 2 (bases 1 to 19104)
AUTHORS Sanchez,A., Kiley,M.P., Klenk,H.D. and Feldmann,H.
TITLE Sequence analysis of the Marburg virus nucleoprotein gene. comparison to Eboia virus and other non-segmented negative-strand RNA viruses
JOURNAL J. Gen. Virol. 73 (Pt 2), 347-357 (1992)
MEDLINE 92166742
REFERENCE 3 (bases 1 to 19104)
AUTHORS Muhlberger,E., Sanchez,A., Randolf,A., Will,C., Kiley,M.P., Klenk,H.D. and Feldmann,H.
TITLE The nucleotide sequence of the L gene of Marburg virus, a filovirus: homologies with paramyxoviruses and rhabdoviruses
JOURNAL Virology 187 (2), 534-547 (1992)
MEDLINE 92188528
REFERENCE 4 (bases 1 to 19104)
AUTHORS Feldmann,H.
TITLE Direct Submission
JOURNAL Submitted (21-MAY-1992) Feldmann H., Zentrum fuer Hygiene der Philipps-Universitaet, Institut fuer Virologie, Robert-Koch-Str. 17, Marburg, Germany, 3550
REMARK revised by [5] MAT
REFERENCE 5 (bases 1 to 19104)
AUTHORS Feldmann,H.
TITLE Direct Submission
JOURNAL Submitted (20-SEP-1994) Feldmann H., Zentrum fuer Hygiene der Philipps-Universitaet, Institut fuer Virologie, Robert-Koch-Str. 17, Marburg, Germany, 3550
REFERENCE 6 (bases 1 to 19104)
AUTHORS Will,C., Muhlberger,E., Linder,D., Slenczka,W., Klenk,H.D. and Feldmann,H.
TITLE Marburg virus gene 4 encodes the virion membrane protein, a type I transmembrane glycoprotein
JOURNAL J. Virol. 67 (3), 1203-1210 (1993)
MEDLINE 93172334
COMMENT On Sep 22, 1994 this sequence version replaced gi:60623.
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PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/40, 15/86, 7/01, C07K 14/08, A61K 39/12

A3

(11) International Publication Number: **WO 00/00616**

(43) International Publication Date: 6 January 2000 (06.01.00)

(21) International Application Number: PCT/US99/14174

(22) International Filing Date: 21 June 1999 (21.06.99)

(30) Priority Data:
60/091,403 29 June 1998 (29.06.98) US

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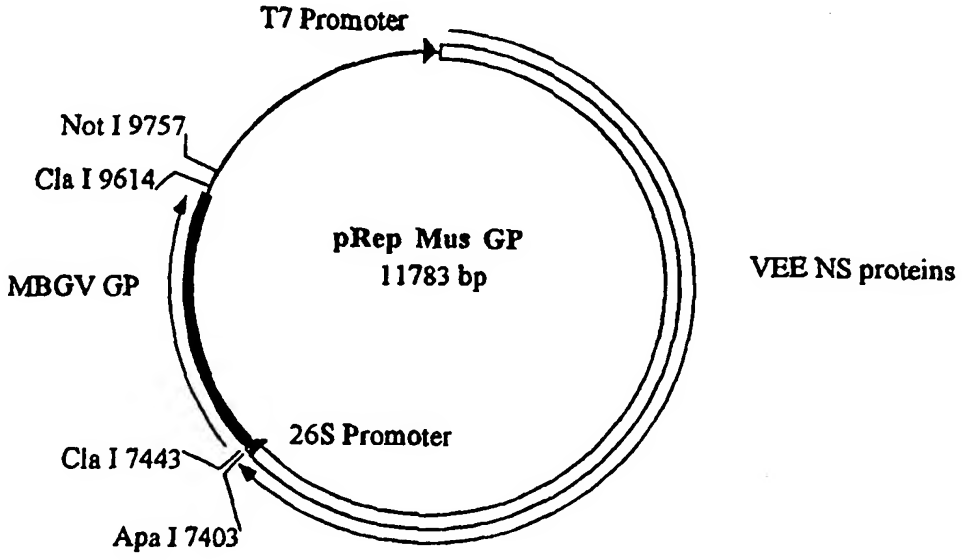
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
6 July 2000 (06.07.00)

(54) Title: MARBURG VIRUS VACCINES



Map of MBGV GP replicon plasmid.

(57) Abstract

Using the MBGV GP, NP and virion proteins, a method and composition for use in inducing an immune response which is protective against infection with MBGV in nonhuman primates is described.

FOR THE PURPOSES OF INFORMATION ONLY

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INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 99/14174

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/40 C12N15/86 C12N7/01 C07K14/08 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 37616 A (UNIV NORTH CAROLINA ;US HEALTH (US); JOHNSTON ROBERT E (US); DAVIS) 28 November 1996 (1996-11-28)	1,2,4,5, 17,19,20
Y	page 8, line 29 -page 9, line 2; claims 1-37	7,14-16

X	C. WILL ET AL.: "Marburg virus gene encodes for the virion mmebrane protein, a type I transmembrane glycoprotein" J. VIROLOGY, vol. 67, no. 3, March 1993 (1993-03), pages 1203-1210, XP002128205 AM.SOC.MICROBIOL.,WASHINGTON,US cited in the application	1-4,6, 17-20
Y	the whole document	5,7, 14-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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20 January 2000	02. 05. 2000
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Internal Application No
PCT/US 99/14174

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A.A. BUKREYEV ET AL.: "The GP-protein of Marburg virus contains the region similar to the 'immunosuppressive domain' of oncogenic retrovirus P15E protein" FEBS LETTERS, vol. 323, no. 1,2, May 1993 (1993-05), pages 183-187, XP002128206 ELSEVIER, AMSTERDAM, NL	1-4,6, 17-20
Y	the whole document	5,7, 14-16

X	M. HEVEY ET AL.: "Antigenicity and vaccine potential of Marburg virus glycoprotein expressed by baculovirus recombinnats" VIROLOGY, vol. 239, 1997, pages 206-216, XP002128207 ACADEMIC PRESS, INC.,NEW YORK, US cited in the application	1-4,6, 17-20
Y	the whole document	5,7, 14-16

X	SANCHEZ A ET AL: "SEQUENCE ANALYSIS OF THE EBOLA VIRUS GENOME: ORGANIZATION, GENETIC ELEMENTS, AND COMPARISON WITH THE GENOME OF MARBURG VIRUS" VIRUS RESEARCH,NL,AMSTERDAM, vol. 29, no. 3, page 215-240 XP000198438 ISSN: 0168-1702	1-4,6, 17-20
Y	figure 8	5,7, 14-16

X	A. SANCHEZ ET AL.: "Variation in the glycoprotein and VP35 genes of Marburg virus strains" VIROLOGY, vol. 240, no. 1, 5 January 1998 (1998-01-05), pages 138-146, XP002128208 ACADEMIC PRESS, INC.,NEW YORK, US	1-4,6, 17-20
Y	the whole document	5,7, 14-16

Y	P. PUSHKO ET AL.: "Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: Expression of heterologous genes in vitro and imunization against heterologous pathogens in vivo" VIROLOGY, vol. 239, 1997, pages 389-401, XP002128209 ACADEMIC PRESS, INC.,NEW YORK, US the whole document	5,7, 14-16

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INTERNATIONAL SEARCH REPORT

Intern: ıl Application No
PCT/US 99/14174

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HEVEY M ET AL: "Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates." VIROLOGY, (1998 NOV 10) 251 (1) 28-37. JOURNAL CODE: XEA., XP002128210 the whole document	1-7, 14-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/14174

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-6,14-20 (partially); 7 (complete)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: (1-6,14-20)-partially; 7-complete

   A recombinant DNA construct comprising (i) a vector, and (ii) at least one MBGV virus DNA fragment encoding GP; the recombinant DNA construct wherein said MBGV virus protein is from strain Musoke; said DNA construct wherein said vector is VEE; host cell transformed with said recombinant DNA construct; said recombinant DNA construct is pRep Mus GP; Self replicating RNA produced from said construct; Infectious alphavirus particles produced from packaging said self replicating RNA; a pharmaceutical composition comprising infectious alphavirus particles; a method for producing MBGV virus proteins;

2. Claims: (1-6,14-20)-partially; 8-complete

   Idem as invention 1 but limited to NP respectively pRep Mus NP;

3. Claims: (1-6,14-20)-partially; 9-complete

   Idem as invention 1 but limited to NVP40 respectively pRep Mus VP40;

4. Claims: (1-6,14-20)-partially; 10-complete

   Idem as invention 1 but limited to VP35 respectively pRep Mus VP35;

5. Claims: (1-6,14-20)-partially; 11-complete

   Idem as invention 1 but limited to VP30 respectively pRep Mus VP30;

6. Claims: (1-6,14-20)-partially; 12-complete

   Idem as invention 1 but limited to VP24 respectively pRep Mus VP24;

7. Claims: (1-6,14-20)-partially; 13-complete

   Idem as invention 1 but limited to GPdeltaTM respectively pRep Mus GPdeltaTM;

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna il Application No
PCT/US 99/14174

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9637616 A	28-11-1996	US 5792462 A	11-08-1998
		AU 5925696 A	11-12-1996
		CA 2220964 A	28-11-1996
		JP 11505719 T	25-05-1999

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 January 2000 (06.01.2000)

PCT

(10) International Publication Number
WO 00/00616 A3

(51) International Patent Classification[7]: C12N 15/40, 15/86, 7/01, C07K 14/08, A61K 39/12

(21) International Application Number: PCT/US99/14174

(22) International Filing Date: 21 June 1999 (21.06.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/091,403 29 June 1998 (29.06.1998) US

(71) Applicant *(for all designated States except US)*: U.S. MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES [US/US]; Dept. of the Army, 504 Scott Street, Frederick, MD 21702 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: HEVEY, Michael, C. [US/US]; 5764 Katsura Court, Frederick, MD 21703 (US). NEGLEY, Diane, L. [US/US]; 8105 Stone Ridge Drive, Frederick, MD 21702 (US). PUSHKO, Peter [US/US]; 917 Seminole Road, Frederick, MD 21701 (US). SMITH, Jonathan, F. [US/US]; 6936 Eylers Valley Flint Road, Sabillasville, MD (US). SCHMALJOHN, Alan, L. [US/US]; 7613 Irongate Drive, Frederick, MD (US).

(74) Agent: HARRIS, Charles, H.; United States Army Medical Research and Material Command, 504 Scott Street, Fort Detrick, MD 21702 (US).

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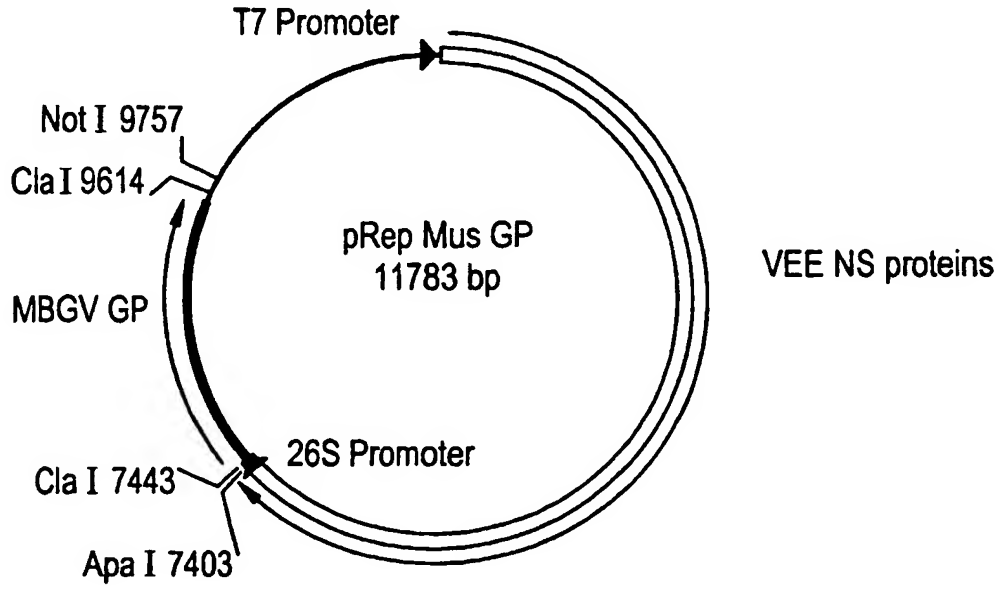
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
6 July 2000

[Continued on next page]

(54) Title: MARBURG VIRUS VACCINES



(57) Abstract: Using the MBGV GP, NP and virion proteins, a method and composition for use in inducing an immune response which is protective against infection with MBGV in nonhuman primates is described.

WO 00/00616 A3

(48) **Date of publication of this corrected version:**
12 April 2001

(15) **Information about Correction:**
see PCT Gazette No. 15/2001 of 12 April 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TITLE OF THE INVENTION

Marburg Virus Vaccines

INTRODUCTION

Marburg virus (MBGV) was first recognized in 1967, when an outbreak of hemorrhagic fever in humans occurred in Germany and Yugoslavia, after the importation of infected monkeys from Uganda (Martini and Siegert, 1971, Marburg Virus Disease. Berlin: Springer-Verlag; Smith *et al.*, 1982, *Lancet* 1, 816-820). Thirty-one cases of MBGV hemorrhagic fever were identified that resulted in seven deaths. The filamentous morphology of the virus was later recognized to be characteristic, not only of additional MBGV isolates, but also of Ebola virus (EBOV) (Johnson *et al.*, 1977, *J. Virol.* 71, 3031-3038; Smith *et al.*, 1982, *Lancet* 1, 816-820; Pattyn *et al.*, 1977, *Lancet* 1, 573-574). MBGV and EBOV are now known to be distinctly different lineages in the family *Filoviridae*, within the viral order Mononegavirales (Kiley *et al.*, 1982, *Intervirology* 18, 24-32; Feldmann and Klenk, 1996, *Adv. Virus Res.* 47, 1-52).

Few natural outbreaks of MBGV disease have been recognized, and all proved self-limiting, with no more than two cycles of human-to-human transmission. However, the actual risks posed by MBGV to global health cannot be assessed because factors which restrict the virus to its unidentified ecological

niche in eastern Africa, and those that limit its transmissibility, remain unknown (Feldmann and Klenk, 1996, supra). Concern about MBGV is further heightened by its known stability and infectivity in aerosol form (Belanov *et al.*, 1996, *Vopr. Virusol.* 41, 32-34; Frolov and Gusev Iu, 1996, *Vopr. Virusol.* 41, 275-277). Thus, laboratory research on MBGV is necessarily performed at the highest level of biocontainment. To minimize future risk, our primary interest has been the identification of appropriate antigens and vaccine strategies that can provide immunity to MBGV.

Early efforts to demonstrate the feasibility of vaccination against MBGV were only partially successful, as inoculation with formalin-inactivated viruses only protected about half the experimental animals (guinea pigs or nonhuman primates) from fatal disease (Ignat'ev *et al.*, 1991, *Vopr. Virusol.* 36, 421-423; Ignat'ev et al., 1996, *J. Biotechnol.* 44, 111-118). We recently demonstrated that the MBGV GP, cloned into a baculovirus vector and expressed as a soluble antigen to be administered in adjuvant, was sufficient to protect most but not all guinea pigs from lethal MBGV challenge (Hevey *et al.*, 1997, *Virology* 239, 206-216). In addition, purified, ^{60}Co-irradiated virus, administered in adjuvant, completely protected guinea pigs from challenge with either of two different strains of MBGV, thus setting a standard for future, more pragmatic, vaccine candidates (Hevey *et al.*, 1997, supra). Experiences with EBOV vaccines have been similar to those with MBGV, reinforcing the difficulties of classical approaches (Lupton *et al.*, 1980, *Lancet* 2, 1294-1295). Recent efforts to develop EBOV vaccines, using three distinctly different approaches (vaccinia recombinants, VEE replicon, and

naked DNA) to achieve viral antigen expression in cells of vaccinated animals, showed that nucleoprotein (NP) as well as GP protected BALB/c mice (VanderZanden *et al.*, 1998, *Virology* 245), whereas protection of guinea pigs by NP was unsuccessful (Gilligan *et al.*, 1997, In: Brown, F., Burton, D., Doherty, P., Mekalanos, J., Norrby, E. (eds). 1997. Vaccines 97 Cold Spring Harbor Press. Cold Spring Harbor, NY; Pushko *et al.*, 1997, In: Brown, F., Burton, D., Doherty, P., Mekalanos, J., Norrby, E. (eds). 1997. Vaccines 97 Cold Spring Harbor Press. Cold Spring Harbor, NY) or equivocal (Xu *et al.*, 1998, Nat. Med. 4, 37-42).

Irrespective of how encouraging filovirus vaccine results may appear in guinea pigs or mice, protection of nonhuman primates is widely taken as the more definitive test of vaccine potential for humans. Low-passage viral isolates from fatal human cases of MBGV or EBOV tend to have uniform lethality in nonhuman primates, but not in guinea pigs or mice. Small animal models with fatal disease outcomes have been achieved only with a subset of filovirus isolates and only then by multiple serial passages in the desired host (Hevey *et al.*, 1997, supra; Connolly *et al.*, 1999, *J. Infect. Dis.* 179, suppl. 1, S203 ; Xu *et al.*, 1998, supra; Bray *et al.*, 1998, *J. Infect. Dis.* 178, 661-665). While highly useful for identification and initial characterization of vaccine candidates, guinea pig and murine models remain somewhat suspect with regard to the possibility that protection in such animals is easier to achieve than in nonhuman primates and, by inference, in humans. For example, with MBGV, peak viremias and viral titers in organs are more than

100 times higher in nonhuman primates than in guinea pigs.

Therefore, there is a need for an efficacious vaccine for MBGV useful for protecting humans against Marburg hemorrhagic fever.

SUMMARY OF THE INVENTION

The present invention satisfies the need discussed above. The present invention relates to a method and composition for use in inducing an immune response which is protective against infection with MBGV.

In this study a vaccine delivery system based on a Venezuelan equine encephalitis (VEE) virus replicon was used to identify candidate protective antigens in nonhuman primates. In this vaccine strategy, a gene coding for a protein of interest is cloned in place of the VEE virus structural genes; the result is a self-replicating RNA molecule that encodes its own replicase and transcriptase functions, and in addition makes abundant quantities of mRNA encoding the foreign protein. When replicon RNA is transfected into eukaryotic cells along with two helper RNAs that express the VEE structural proteins (glycoproteins and nucleocapsid), the replicon RNA is packaged into VEE virus-like particles by the VEE virus structural proteins, which are provided in trans. Since the helper RNAs lack packaging signals neccessary for further propagation, the resulting VEE replicon particles (VRPs) which are produced are infectious for one cycle but are defective thereafter. Upon infection of an individual cell with a VRP, an abortive infection occurs in which the infected cell produces the protein of interest in abundance, is

ultimately killed by the infection, but does not produce any viral progeny (Pushko *et al.*, 1997, *Virology* 239, 389-401). The VEE replicon is described in greater detail in U.S. Patent No. 5,792,462 issued to Johnston *et al.* on August 11, 1998.

Results shown here demonstrate that the VEE replicon is a potent tool for vaccination with MBGV antigens. Guinea pigs were protected by vaccination with packaged replicons that expressed GP, or by either of two replicons which expressed internal MBGV antigens (NP and VP35). GP expressed from the VEE replicon elicited an even more robust immunity than was achieved previously with a baculovirus-produced soluble GP administered in adjuvant. When results were extended to nonhuman primates, complete protection with GP was demonstrated. The data shown here constitute the most emphatic proof to date that an efficacious vaccine for MBGV is feasible, and define candidate antigens for such a vaccine.

Therefore, it is one object of the present invention to provide a VEE virus replicon vector comprising a VEE virus replicon and a DNA fragment encoding any of the MBGV GP, NP, VP40, VP35, VP30, and VP24, and GPΔTM, a GP deletion mutant from which the C-terminal 39 amino acids encoding the transmembrane region and cytoplasmic tail of MBGV GP were removed.

It is another object of the present invention to provide a self replicating RNA comprising the VEE virus replicon and any of the MBGV GP, GPΔTM, NP, VP40, VP35, VP30, and VP24 described above.

It is another object of the present invention to provide infectious VEE virus replicon particles produced from the VEE virus replicon RNA described above.

It is further an object of the invention to provide an immunological composition for the protection of mammals against MBGV infection comprising VEE virus replicon particles containing nucleic acids encoding any of the MBGV GP, GPΔTM, NP, VP40, VP35, VP30, and VP24 or a combination of different VEE virus replicons each containing nucleic acids encoding a different MBGV protein from any of MBGV GP, GPΔTM, NP, VP40, VP35, VP30, and VP24.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. Indirect immunofluorescence of Vero cells infected with packaged VEE replicons expressing the indicated antigens.

Figure 2. Immunoprecipitation of MBGV proteins expressed from an alphavirus replicon in Vero cells using convalescent guinea pig polyclonal anti-MBGV serum. Lane 1, cell lysate from Vero cells infected with MBGV GP replicon; lane 2, cell lysate from Vero cells infected with MBGV GPΔTM replicon; lane 3, supernatant from Vero cells infected with MBGV GPΔTM replicon; lanes 4-6, cell lysate from Vero cells infected with various clones of MBGV NP replicon; lanes 7-8, cell lysate from Vero cells infected with

various clones of MBGV VP40 replicon; lane 9, sucrose gradient-purified ^{35}S-labeled MBGV, * an unidentified 46-50 KDa protein observed in virion preparations.

Figure 3. Anti-MBGV ELISA titers of cynomolgus monkeys after three inoculations with recombinant replicon 17 days before or after challenge with MBGV. Prechallenge samples were obtained 17 days before challenge, while postchallenge samples were obtained 17 days after challenge. GP, animals inoculated with VEE replicons expressing MBGV GP; NP, animals inoculated with VEE replicon expressing MBGV NP; GP+NP, animals inoculated with a mixture of VEE replicons expressing either MBGV GP or NP; Flu HA, animals inoculated with VEE replicon expressing influenza HA. Numbers inside each symbol represent the same individual in each group. Symbols filled in with cross hatch marks signify animals that died from infection.

Figure 4. Viremia level in cynomolgus monkeys inoculated with alphavirus replicons followed by challenge with MBGV (Musoke). ● Animals vaccinated with VEE replicons expressing MBGV GP; ◆ animals vaccinated with VEE replicons expressing MBGV NP; ■, animals vaccinated with a mixture of VEE replicons which expressed either MBGV GP or NP; Δ, animals vaccinated with VEE replicons expressing influenza HA. Open symbols represent animals that died. Closed symbols represent animals that lived. Dotted line notes the lower limit of detection of this plaque assay (1.7Log_{10} PFU/ml).

Figure 5. Serum AST levels in VEE replicon inoculated cynomolgus macaques after challenge with MBGV (Musoke). ● The one animal (of six) vaccinated with VEE replicons expressing MBGV GP that exhibited AST abnormality at any time point. ◆, animals vaccinated with VEE replicons expressing MBGV NP; Δ, animals vaccinated with VEE replicon expressing influenza HA. Open symbols represent animals that died. Closed symbols represent animals that lived. Dotted line demarks 88 U/L, which is the mean (38 U/L) plus three standard deviations of pre-bleed values from the 12 monkeys in this experiment.

Figure 6: Schematic of pRep Mus GP.

Figure 7: Schematic of pRep Mus GPΔTM.

Figure 8: Schematic of pRep Mus NP.

Figure 9: Schematic of pRep Mus VP40.

Figure 10: Schematic of pRep Mus VP35.

Figure 11: Schematic of pRep Mus VP30.

Figure 12: Schematic of pRep Mus VP24.

DETAILED DESCRIPTION

In the description that follows, a number of terms used in recombinant DNA, virology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Filoviruses. The filoviruses (e.g. Marburg virus, MBGV) cause acute hemorrhagic fever characterized by high mortality. Humans can contract filoviruses by infection in endemic regions, by contact with imported primates, and by performing

scientific research with the virus. However, there currently are no available vaccines or effective therapeutic treatments for filovirus infection. The virions of filoviruses contain seven proteins which include a surface glycoprotein (GP), a nucleoprotein (NP), an RNA-dependent RNA polymerase (L), and four virion structural proteins (VP24, VP30, VP35, and VP40). Little is known about the biological functions of these proteins and it is not known which antigens significantly contribute to protection and should therefore be used to induce an immune response by an eventual vaccine candidate.

Replicon. A replicon is equivalent to a full length virus from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site. Transcription of the RNA from the replicon yields an RNA capable of initiating infection of the cell identical to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA in *trans*. This is typically done with two helpers also called defective helper RNAs. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for

subgenomic mRNA transcription and the sequences for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the cell. The particles can then by inoculated into animals similar to parent virus. The replicon particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only the viral nostructural proteins and the product of the heterologous gene cloned in place to the structural proteins.

The VEE virus replicon is a genetically reorganized version of the VEE virus genome in which the structural proteins genes are replaced with a gene from an immunogen of interest, in this invention, the MBGV virion proteins. The result is a self replicating RNA (replicon) that can be packaged into infectious particles using defective helper RNAs that encode the glycoprotein and capsid proteins of the VEE virus.

Subject. Includes both human, animal, e.g., horse, donkey, pig, guinea pig, mouse, hamster, monkey, chicken, bats, birds and insects such as mosquito.

In one embodiment, the present invention relates to a recombinant DNA molecule that includes a VEE replicon and a DNA sequence encoding any of MBGV

virion proteins GP, GPΔTM, NP, VP40, VP35, VP30, VP24. The sequences encoding the Marburg proteins GP, GPΔTM, NP, VP40, VP35, VP30, VP24 corresponding to nucleotides 104-11242 of the Genbank sequence is presented in SEQ ID NO:1; the GP DNA fragment extends from nucleotide 5932 to 8033, of which nucleotides 5940-7985 encode the protein identified in SEQ ID NO:2; the GPΔTM DNA fragment, a GP deletion mutant from which the C-terminal 39 amino acids encoding the transmembrane region and cytoplasmic tail of MBGV GP were removed, extends from nucleotides 5933 to 7869, of which nucleotides 5940-7871 encode the protein; NP, identified in SEQ ID NO:3, is encoded by the DNA fragment extending from nucleotides 104 to 2195; VP40 DNA fragment extends from nucleotide 4564 to 5958, of which nucleotides 4567-5416 encode the protein identified in SEQ ID NO:4; VP35 DNA fragment extends from nucleotide 2938 to 4336, of which nucleotides 2944-3933 encode the protein identified in SEQ ID NO:5; VP30 DNA fragment extends from nucleotide 8861 to 9979, of which nucleotides 8864-9697 encode the protein identified in SEQ ID NO:6; VP24 DNA fragment extends from nucleotide 10182 to 11242, of which nucleotides 10200-10961 encode the protein identified in SEQ ID NO:7.

When the DNA sequences described above are in a replicon expression system, such as the VEE replicon described above, the proteins can be expressed *in vivo*. The DNA sequence for any of the MBGV virion proteins described above can be cloned into the multiple cloning site of a replicon such that transcription of the RNA from the replicon yields an infectious RNA containing the sequence(s) which encodes the MBGV virion protein or proteins of interest. Use of helper RNA containing sequences

necessary for encapsulation of the viral transcript will result in the production of viral particles containing replicon RNA which are able to infect a host and initiate a single round of replication resulting in the expression of the MBGV virion proteins. Such replicon constructs include, for example, VP24 cloned into a VEE replicon, pRep Mus VP24, VP30 cloned into a VEE replicon, pRep Mus VP30, VP35 cloned into a VEE replicon, pRep Mus VP35, and VP40 cloned into a VEE replicon, pRep Mus VP40, NP cloned into a VEE replicon, pRep Mus NP, GP cloned into a VEE replicon, pRep Mus GP, GPΔTM cloned into a VEE replicon, pRep Mus GPΔTM. The sequences encoding the MBGV proteins were cloned into the replicon vector by methods known in the art and described below in Materials and Methods. Schematic diagrams of the resulting constructs are shown in the Figures. The VEE constructs containing Marburg proteins can be used as a DNA vaccine, or for the production of RNA molecules as described below.

In another embodiment, the present invention relates to RNA molecules resulting from the transcription of the constructs described above. The RNA molecules can be prepared by *in vitro* transcription using methods known in the art and described in the Examples below. Alternatively, the RNA molecules can be produced by transcription of the constructs *in vivo*, and isolating the RNA. These and other methods for obtaining RNA transcripts of the constructs are known in the art. Please see Current Protocols in Molecular Biology. Frederick M. Ausubel et al. (eds.), John Wiley and Sons, Inc. The RNA molecules can be used, for example, as a direct RNA vaccine, or to transfect cells along with RNA from helper plasmids, one of which expresses VEE

glycoproteins and the other VEE capsid proteins, as described above, in order to obtain replicon particles.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to mouse and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of MBGV virion proteins, such as glutathione S-transferase. The recombinant molecule can be suitable for transfecting eukaryotic cells, for

example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as CHO cells, vero cells, and COS cells to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art.

The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein cloned into the VEE replicon, or a source of RNA transcribed from the replicon as described above, or a source of replicon particles.

In a further embodiment, the present invention relates to a method of producing the recombinant or fusion protein which includes culturing the above-described host cells, under conditions such that the DNA fragment is expressed and the recombinant or fusion protein is produced thereby. The recombinant or fusion protein can then be isolated using methodology well known in the art. The recombinant or

fusion protein can be used as a vaccine for immunity against infection with MBGV or as a diagnostic tool for detection of MBGV infection. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit MBGV virus function, such as host proteins or chemically derived agents or other proteins which may interact with the virus to inhibit its replication or survival.

In another embodiment, the present invention relates to a MBGV vaccine comprising one or more replicon particles derived from one or more replicons encoding one or more MBGV virion proteins. The present invention relates to a method for providing immunity against MBGV virus said method comprising administering one or more replicon particles containing any combination of the MBGV virion proteins to a subject such that a protective immune reaction is generated. Even though the MBGV strain Musoke was used in the examples below, it is expected that protection would be afforded using virion proteins from other MBGV strains, as well as significant cross protection between strains.

Vaccine formulations of the present invention comprise an immunogenic amount of a replicon particle, resulting from one of the replicon constructs described above, or a combination of replicon particles as a multivalent vaccine, in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered. An amount of from about 10^5 to 10^8 or more replicon particles per dose with one to three doses one month apart is suitable, depending upon the age and species of the subject being treated. Exemplary

pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the replicon particles disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, orally and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the replicon as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed.

When the replicon RNA or DNA is used as a vaccine, the replicon RNA or DNA can be administered directly using techniques such as delivery on gold beads (gene gun), delivery by liposomes, or direct injection, among other methods known to people in the art. Any one or more constructs or replicating RNA described above can be use in any combination effective to illicit an immunogenic response in a subject. Generally, the nucleic acid vaccine administered may be in an amount of about 1-5 ug of

nucleic acid per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

The following MATERIALS AND METHODS were used in the examples that follow.

Cell cultures and viruses

Vero E6 (Vero C1008, ATCC CRL 1586), Vero 76 (ATCC CRL 1587), and BHK (ATCC CCL 10) cells were grown in minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum and gentamicin (50 µg/ml). MBGV (strain Musoke) was isolated from a human case in 1980 in Kenya (Smith *et al.*, 1982, *Lancet* 1, 816-820), and a derivative of this virus (six passages in Vero 76 cells) was used to challenge the cynomolgus monkeys. The MBGV (Musoke) that was adapted for guinea pig lethality and plaque-

picked three times was described previously (Hevey *et al.*, 1997, *Virology* 239, 206-210).

Construction of recombinant VEE replicons

MBGV gene clones pGem-GP, pGem-NP, pTM1-VP40, pTM1-VP35, pTM1-VP30, and pTM1-VP24 were generously provided by Heinz Feldmann and Anthony Sanchez (Centers for Disease Control and Prevention, Atlanta, GA) (Will et al., 1993, J. Virol. 67, 1203-1210; Sanchez et al., 1992, J. Gen. Virol. 73, 347-357; Feldman et al., 1992, Virus Res. 24, 1-19). VEE replicon and shuttle vector as well as the replicons that express Lassa virus NP and Flu HA were previously described (Pushko *et al.*, 1997, *Virology* 239, 289-401). The MBGV GP gene from pGem-GP was excised with Sal I and subcloned into the Sal I site of the shuttle vector by using standard techniques (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. 2 ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor). A clone with the MBGV GP gene in the correct orientation was excised with Apa I and Not I and this fragment was cloned into the Apa I and Not I sites of the VEE replicon plasmid.

Construction of pBluescript-KS(+)-GPΔTM, a deletion mutant of MBGV from which the C-terminal 39 amino acids (transmembrane region and cytoplasmic tail) of MBGV GP were removed, was previously described (Hevey *et al.*, 1997, supra). Here, the MBGV GPΔTM gene was excised from pBluescript-KS(+) with Hind III, and the resulting fragment ligated into the Hind III site of the shuttle vector. MBGV GPΔTM gene was excised from the shuttle vector using Cla I, and the resulting fragment ligated into the VEE replicon plasmid.

The MBGV NP gene was amplified by PCR performed with 1 ng of pGem NP as template DNA, 1 µg each of

forward (5'-CCG ACC ATG GAT TTA CAC AGT TTG TTG G-3', SEQ ID NO:8) and reverse primer (5'-CTA GCC ATG GCT GGA CTA CAA GTT CAT CGC-3' SEQ ID NO:9), and AmpliTaq polymerase (GeneAmp PCR reagent kit, Perkin Elmer, Branchburg, NJ). The primers contained an NcoI recognition sequence at the 3' terminus end (5-10 inclusive for both the forward and reverse primers). The reaction conditions were: 40 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 1 min., followed by a final extension step at 72°C for 5 min. The product was cloned into the pCR™II (InVitrogen, Carlsbad, CA) vector, excized with Eco RI, then subcloned into the shuttle vector using Eco RI sites. The MBGV NP gene was excised with Cla I and ligated into the VEE replicon plasmid.

The MBGV VP40, VP35, VP30, and VP24 genes were excised from pTM1 with Bam HI and ligated into the Bam HI site of the shuttle vector. These MBGV genes were then excised from shuttle vectors using either Cla I (VP35, VP30, and VP24) or Apa I and Not I (VP40) and ligated into the VEE replicon plasmid.

Packaging of replicons into VEE-like particles and determination of replicon titer

Replicon RNAs were packaged into VRPs as described previously (Pushko *et al.*, 1997, *Virology* 239, 389-401). Briefly, BHK cells were cotransfected with RNA transcribed *in vitro* from the replicon plasmid and from two helper plasmids, one of which expressed VEE glycoproteins and the other VEE capsid protein. The cell culture supernatant was harvested approximately 30 h after transfection and the replicon particles were concentrated and partially purified by pelleting through a 20% sucrose cushion (SW28 rotor, 25,000 rpm, 4 h), after which they were resuspending in 1 ml PBS. To assay titers of packaged replicons,

Vero cells (10^5 cells per well in eight-chamber slides, Labtek slides, Nunc Inc.) were infected with serial dilutions of the replicon particles and incubated for 16-18 h at 37°C to allow for expression of the MBGV genes. After rinsing and fixating with acetone, antigen-positive cells were identified by indirect immunofluorescence assay (IFA) as described previously (Schmaljohn *et al*, 1995, *Virology* 206, 963-972). The antibodies used included MAbs specific for MBGV GP (II-7C11), NP (III-5F8), VP40 (III-1H11), VP35 (XBC04-BG06), and VP30 (III-5F11 and 5F12) (Hevey *et al.*, 1997, supra). To detect VP24 antigen, a monkey anti-MBGV serum was used, a monkey anti-Lassa serum was used to detect expression of Lassa NP in cells, and influenza HA was detected with serum from a mouse immunized with a VEE replicon expressing influenza HA (provided by Dr. Mary Kate Hart, USAMRIID).

Immunoprecipitation and gel electrophoresis of proteins expressed by VEE replicons

Expressed MBGV antigens were immunoprecipitated and analyzed by gel electrophoresis as described previously (Hevey *et al.*, 1997, supra). Briefly, Vero cells were infected (MOI ≥ 3) with VRP expressing a single MBGV antigen. Complete medium was replaced 16-18 h postinfection by methionine- and cysteine-free medium for 1 h, and monolayers were then labeled with ^{35}S-methionine and cysteine for 4 h. Convalescent guinea pig anti-MBGV (group 1, Table 5, in Hevey *et al.*, 1997, supra) was used to immunoprecipitate MBGV-specific proteins from the resulting cell lysates.

Vaccination of guinea pigs with VEE replicons expressing MBGV proteins

Inbred strain 13 guinea pigs (maintained as a colony at USAMRIID) were inoculated subcutaneously

with 10^6 focus-forming units (FFU) of VRP in a total volume of 0.5 ml administered at two dorsal sites. Guinea pigs were anesthetized, bled, and those that received two or three doses of replicon inoculated (as described for the first vaccine dose) 28 days after the primary vaccination. Guinea pigs were anesthetized and bled again 28 days later, and animals that received three doses of replicons were inoculated, as described above. Animals were anesthetized and bled 21 days later, and challenged 7 days after the last bleed with $10^{3.0}$ plaque forming units (PFU) (ca. 2000 LD_{50}) guinea pig adapted MBGV. Animals were examined daily for signs of illness. Heparinized plasma was obtained from the retroorbital sinus of anesthetized animals 7 days postinfection for assay of viremia. Surviving guinea pigs were observed for at least 30 days after challenge, then anesthetized and exsanguinated. Viremia titers was measured by plaque assay on Vero E6 cells.

Vaccination of cynomolgus monkeys with replicons

Twelve cynomolgus macaques (*Macaca fascicularis*), 11 females and 1 male, ranging from 2.8 to 4.5 Kg, were inoculated subcutaneously with 10^7 FFU of VRP in a total volume of 0.5 ml at one site. Monkeys were anesthetized with ketamine, bled, and inoculated (as described for the first vaccine dose) 28 days after the primary injection, and again 28 days after the second. Animals were anesthetized and bled 21 days after the third vaccine dose, then were challenged 14 days later with $10^{3.9}$ PFU MBGV subcutaneously. Here and in guinea pig experiments, the inoculum was back-titrated to ensure proper dose delivery. Animals were examined daily by the attending veterinarian for signs

of illness, and given buprenorphine (Buprenex) at a dosage of 0.01 mg/kg body weight, to provide analgesia upon signs of distress. Of the unprotected animals, three succumbed abruptly, while one was euthanized in extremis. A detailed clinical evaluation, serum for viremia determination and blood chemistries, as well as EDTA blood was obtained from anesthetized animals 17 days before and 3, 5, 7, 10, 17, and 32 days postinfection. Viremia was measured by plaque assay on Vero E6 cells.

MBGV ELISA and infectivity assays

Antibody titers in guinea pig plasmas or monkey sera were determined by an indirect ELISA as described previously (Hevey *et al.*, 1997, supra). Briefly, antigen consisting of purified, irradiated virus was coated directly onto PVC plates and serial dilutions of test serum were added to wells containing antigen. The presence of bound antibody was detected by use of the appropriate horseradish peroxidase conjugated anti-species antibody (HPO-goat-anti-guinea pig IgG H+L; HPO-goat-anti-monkey IgG H+L). Endpoint of reactivity was defined as the dilution at which OD_{405} was 0.2 as determined by extrapolation of a four parameter curve fit (SOFTmax®, Molecular Devices Corp. Sunnydale, CA) of background-subtracted mean OD versus dilution. Results shown in any table or figure are from a single assay to allow more valid comparison of endpoints. Plaque assays were performed on Vero E6 cells with a semi-solid overlay on serial dilutions of samples. Viral plaques were visualized by staining viable cells with neutral red 6-7 days postinfection. To measure plaque reduction neutralization, equal volumes of a virus stock (target plaque dose was 100 PFU) and serum diluted in cell culture medium were mixed and incubated at 37°C for 1 h. The resulting

sample was assayed by plaque assay on Vero E6 cells for more than a 50 % reduction in PFU compared to control samples.

Clinical laboratory assays

For nonhuman primate studies, hematological results were obtained with a Coulter instrument, and differential counts were performed manually. Clinical chemistry results were obtained with a Piccolo™ analyzer (Abaxis, Inc., Sunnydale, CA) using the diagnostic panel General Chemistry 12, which measures alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), amylase, aspartate aminotransferase (AST), calcium, cholesterol, creatinine, glucose, total bilirubin, total protein, and urea nitrogen.

Example 1

Analysis of protein products synthesized after infection of Vero cells with VEE replicons that expressed MBGV proteins

Results of indirect immunofluorescence assay (IFA) analyses of Vero cells infected with different recombinant VEE replicons expressing MBGV proteins, are shown in Figure 1. Expression of the indicated protein products was detected both with polyclonal guinea pig anti-MBGV and with monoclonal antibodies (MAbs) specific for the indicated MBGV proteins or, in the case of VP24 (for which no MAbs were available), with convalescent serum from a monkey that had survived infection with MBGV. There were distinct staining patterns for several of the expressed proteins. MBGV GP was observed as a plasma membrane fluorescence, while the GPΔTM provided a more diffuse cytoplasmic staining. These different staining patterns were not unexpected as GPΔTM, which lacks the hydrophobic transmembrane region of GP, is a secreted product. MBGV NP and VP35 formed discrete patterns in

the cytoplasm of cells. MBGV VP40 demonstrated a more diffuse cytoplasmic staining pattern. MBGV VP30 was present in unique large globules staining in the cytoplasm of cells. MBGV VP24 staining was typically perinuclear. In summary, IFA served to assure that the appropriate antigen was expressed in a given preparation; it highlighted staining patterns, which demonstrated the localization of the expressed MBGV proteins in Vero cells; and it served as the basis for the assay whereby 10-fold dilutions of VRPs were quantitated for infectivity, as focus forming units (FFU).

Expression, antigenicity, and size determination of the MBGV proteins were confirmed by immunoprecipitation and gel electrophoresis. The results obtained from expression of MBGV GP, GPΔTM, NP, and VP40 in Vero cells are shown in Figure 2. Products of the expected sizes were specifically immunoprecipitated from replicon-infected cell lysates. Glycosylation of MBGV GP more than doubles the predicted size of the peptide chain, and typically results in a heterogeneous array of posttranslationally modified products (Feldmann *et al.*, 1991, *Virology* 182, 353-356; Feldman et al., 1994, *Virology* 199, 469-473), especially in GP from cell lysates, as shown in Figure 2, lane 1. As expected and shown previously in the baculovirus system, GPΔTM was secreted, and thus present in the supernatant of replicon-infected cells (Fig. 2, Lane 3). Appropriately, both the cell-associated (lane 2) and secreted (lane 3) forms of GPΔTM appeared smaller than the largest forms of GP (lane 1). The secreted form of GPΔTM appeared larger and somewhat more homogeneous than the same molecule from cell lysates, as noted previously (Hevey *et al.*, 1997, supra)

(compare Fig. 2, Lanes 2 and 3). This difference likely reflects the more complete glycosylation of the secreted product compared to partially glycosylated forms of this protein expected to be present in the cell. In this gel, and with considerably less intensity in other preparations, an unidentified protein of approximately 46 KDa, which can be immunoprecipitated with GP-specific monoclonal antibodies (not shown), is evident in MBGV virions (Fig. 2, Lane 9). Although it remains to be confirmed, this product may be the glycosylated form of a putative 27 KDa cleavage product of GP, reported to be the result of a posttranslational, furin-mediated cleavage of GP (Volchkov *et al*, 1998, *Proc. Natl. Acad. Sci. USA* 95:5762-5767). Replicon-expressed MBGV NP (Fig. 2, Lanes 4-6) and VP40 (Fig. 2, Lanes 7-8) comigrated with the authentic proteins present in purified MBGV virions. In other experiments, the reactivity with polyclonal or MAbs and the authentic electrophoretic migrations of the remaining replicon-expressed MBGV proteins (VP30, VP35, and VP24) were similarly demonstrated (data not shown).

Example 2

Protective efficacy of VEE replicons expressing MBGV proteins in strain 13 guinea pigs

Groups of strain 13 guinea pigs were inoculated with packaged recombinant VEE replicons expressing individual MBGV proteins, and later challenged with $10^{3.3}$ LD_{50} guinea pig-adapted MBGV subcutaneously. Results are shown in Table 1. MBGV GP protected guinea pigs from both death and viremia when administered as a three dose regimen. In addition, no reduction in efficacy or potency was observed when a

two dose regimen was instituted, and significant efficacy was observed even when a single dose of 10^6 FFU of VRP expressing MBGV GP was used as an immunogen. The efficacy of either the two or three dose vaccine schedule was further demonstrated by the observation that no boost in postchallenge ELISA titers were observed. This result suggested minimal antigen exposure after challenge with MBGV, and thus robust or even sterile immunity in these animals. MBGV GPΔTM, which was previously shown to be protective as a vaccine when produced from insect cells, also protected guinea pigs from death and viremia when delivered in an VEE virus replicon. Again, there were no increases in postchallenge ELISA titers in the group of animals immunized with GPΔTM, thus no differences were discerned in the vaccine efficacy of membrane-bound versus soluble GP.

Table 1

Protection of replicon inoculated strain 13 guinea pigs from lethal challenge with Marburg virus (Musoke isolate)

		Log 10 ELISA Titer*				
	# of Doses					
Antigen	Replicon	S/T[a]	Day-7	Day 64	Viremia[b]	V/T[c]	MDD
GP	3	6/6**	4.21	3.80	<1.7	0/6	-
GP	2	6/7**	4.30	4.06	<1.7	0/6	-
GP	1	5/6*	2.89	4.19	4.1	1/6	9
NP	3	6/6**	3.38	3.94	<1.7	0/6	-
VP40	3	1/6	2.83	2.68	4.5	5/6	10
GPΔTM	3	6/6**	3.93	3.65	<1.7	0/6	-
VP35	3	5/6*	1.99	3.75	3.7	5/6	13
VP30	3	0/6	2.23	-	5.8	6/6	10
VP24	3	1/6	<1.5	4.31	5.6	6/6	11
Lassa NP	3	1/6	<1.5	4.19	6.0	5/6	10
None	-	1/6	<1.5	4.25	5.2	5/6	11

*Endpoint titer of equal volumes of serum pooled from animals in each group against MBGV Musoke

[a] Survivors/Total (S/T) on day 30 postinfection. ** indicates $p<0.01$, * indicates $p<0.05$.

[b] Viremia (Log_{10} PFU/ml) day 7 postinfection. Where ≥2 animals were viremic, a GMT was calculated.

[c] Viremic animals/total (V/T) on day 7 postinfection. All animals that died were viremic.

In the experiment shown, MBGV NP protected all vaccinated guinea pigs from both viremia and death, while MBGV VP35 vaccination resulted in five of six animals surviving, but four of the five survivors were viremic seven days postinfection. None of the other MBGV viral proteins cloned into VEE replicons evoked significant protection against a lethal challenge with MBGV. Thus, the proteins that showed the most promise as vaccine candidates in the guinea pig model were MBGV GP and NP. Cumulative results from this and additional experiments (not shown) in strain 13 guinea pigs inoculated three times with VRPs demonstrated complete survival with GP (18/18), and less complete protection with NP (16/18) and VP35 (13/18) as compared with controls (2/24).

Example 3

Protection of cynomolgus monkeys vaccinated with recombinant VEE replicons expressing either MBGV GP and/or NP

Encouraged by the success in vaccinating guinea pigs against MBGV, we evaluated the ability of these same VEE replicons to protect cynomolgus macaques from lethal MBGV infection. The monkeys received 10-fold higher doses of replicons, but on an identical schedule as tested in the guinea pigs. Four groups contained three monkeys each. One group received VRPs which expressed MBGV GP; a second group received VRPs

which expressed MBGV NP; a third group received a mixture of MBGV GP and MBGV NP VRPs; and a fourth received VRPs which expressed a control antigen (influenza HA) irrelevant to MBGV immunity. Anti-MBGV ELISA antibody titers were monitored throughout the experiment.

All animals that received VEE replicons expressing MBGV GP, either alone or in combination with MBGV NP, survived challenge with 8000 PFU MBGV without any observed signs of illness (Table 2). Of the three animals vaccinated with MBGV NP, one died 8 days after challenge from MBGV disease. The other two NP recipients displayed signs of illness 7-9 days after challenge, but eventually recovered. One NP-inoculated survivor had a relatively mild disease (slightly reduced activity and responsiveness), while the other had severe disease which included obvious petechiae, loss of weight, reduced activity, and fever. All control animals succumbed, with clinical signs first noted on day 7 or 8, and deaths occurring on days 9 or 10 postchallenge.

Table 2

Survival of replicon-inoculated cynomolgus monkeys•

Replicon[a]	Survival/Total	Sick/Total	Day of Death
GP	3/3*	0/3	-
NP	2/3	3/3	8
GP + NP	3/3*	0/3	-
Influenza HA	0/3	3/3	9,9,10

• surviving animals remain healty >90 days postchallenge.
[a] Antigen delivered by VEE replicon.
* Indicates p=0.05.

The pre- and postchallenge ELISA antibody titers of the cynomolgus macaques are shown in Figure 3. All

animals inoculated with replicons that expressed MBGV proteins demonstrated prechallenge ELISA titers to purified MBGV antigen. Of the three GP-vaccinated animals that survived challenge, two demonstrated a modest boost in ELISA antibody titer (10-30 fold) when pre- and postchallenge samples were compared. The two surviving NP-inoculated macaques had larger boosts in ELISA antibody titers (100-300 fold) when pre- and postchallenge samples were compared. Two of three animals vaccinated with both GP and NP also demonstrated 100- to 300-fold rise in ELISA titers. These observations, in conjuction with the back titration of the MBGV challenge inoculum (8000 PFU), confirmed that all groups were unambiguously challenged, and that two monkeys had particularly robust immunity that apparently restricted virus replication below an immunogenic threshold.

A plaque reduction neutralization assay was performed on pre- and postchallenge serum samples. No neutralization activity was observed, at 1:20 or higher dilutions, in any sample. It should be noted that it is frequently difficult to demonstrate filovirus neutralizing antibody *in vitro*; however, antibodies may nonetheless be relevant *in vivo* (Hevey *et al.*, 1997, *Virology* 239, 206-216), perhaps via mechanisms other than classical neutralization (Schmaljohn *et al.*, 1982, supra).

The viremia levels in each of the monkeys at several time points after MBGV challenge are shown in Figure 4. The data illustrate the profound differences between lethally infected control animals and healthy survivors. Most striking, none of the animals vaccinated with GP, either alone or in combination with NP, had infectious MBGV virus in their sera that was detectable by plaque assay.

Animals vaccinated with a replicon expressing influenza HA were all viremic by day 3 postchallenge and demonstrated sharp rises in MBGV viremia levels which peaked at 7.5-8.0 Log_{10} PFU/ml on day 7 postinfection. Among monkeys vaccinated with NP, one died with viremias indistinguishable from controls. In contrast, the two NP-vaccinated monkeys that recovered had peak viremias that were diminished ≥1000 fold compared with controls. By day 10 postinfection, the NP-vaccinated monkey with the milder illness had no detectable viremia, while the more severely affected monkey still had ~ 4.5 Log_{10} PFU/ml virus. By day 17 postinfection no viremia was detectable in either of the surviving NP vaccinated animals.

Example 4

Additional measures of vaccine-mediated protection

Upon necropsy of the control and the unprotected NP-inoculated monkeys, MBGV titers in their livers were 9.2, 9.7, 9.4, and 9.6 Log_{10} PFU/gm. Virus was detected in all other organs examined as well, and although abundant, was at least 10-fold lower than in the liver. Not surprisingly, elevated liver enzymes were the most obvious abnormal feature in clinical chemistries. As shown in Figure 5, unprotected monkeys had elevated AST levels by day 5 or 7 postinfection, and these were paralleled by similarly profound increases in ALT and ALP (not shown). Terminal samples were automatically rejected by the instrument as too lipemic or hemolyzed; however, in a previous set of control monkeys liver enzymes had continued to ascend dramatically (not shown). With regard to vaccine-mediated protection, it is instructive that the two NP-inoculated survivors

exhibited marked but transient rises in their liver enzymes (Fig. 5), which is consistent with their viremias and signs of MBGV disease. Also, the more severely affected NP- inoculated survivor exhibited a transient rise in urea nitrogen and creatinine (not shown), coincident with recovery and viral clearance. This may have been due to virus-antibody complexes perturbing kidney function, or to direct viral damage to the organs. In contrast, the six monkeys vaccinated with GP exhibited either a minimal rise at one time point (i.e., the one GP animal shown in Fig. 5) or no significant increases in liver enzymes at any time evaluated. Other clinical chemistries and hematological findings remained normal in MBGV-inoculated macaques vaccinated previously with GP or GP+NP, in contrast with control monkeys that exhibited the expected profound end-stage abnormalities in both hematological and chemistry measurements (Johnson *et al.*, 1995, *Int. J. Exp. Pathol.* 76, 227-236).

Discussion

To our knowledge, this is the first report of any filovirus vaccine shown to be completely efficacious in nonhuman primates. Before these observations, we were cautiously optimistic about the overall feasibility of an efficacious vaccine for MBGV, but were also concerned that proofs of filovirus vaccine concepts in guinea pigs may not necessarily forecast success in nonhuman primates and, by inference, in humans. Results presented here defined GP, possibly in combination with NP, as candidate antigens for a MBGV vaccine, and demonstrated that nearly complete immunity is achievable in nonhuman primates.

We chose an alphavirus replicon based on VEE virus to deliver the antigens of interest. This method of vaccination has several advantages (Pushko

et al., 1997, *Virology* 239, 389-401), including the ability to produce large quantities of antigen *in situ*, so that native processing of the antigens might evoke a broad array of immune responses. In addition, all transcription of RNA occurs in the cytoplasm of cells, which avoids RNA splicing problems sometimes observed when proteins of RNA viruses are expressed from the nucleus. Moreover, VEE replicons have proven stable after packaging into VRPs. In addition to robust antibody induction, alphavirus replicons have been demonstrated to elicit cytotoxic T lymphocytes in mice (Caley *et al.*, 1997, *J. Virol.* 71, 3031-3038; Zhou *et al.*, 1995, *Proc. Natl. Acad. Sci. U.S.A.* 92, 3009-3013). The success reported here using VEE replicons to vaccinate monkeys against lethal MBGV challenge justifies a more detailed analysis of the potential of these vectors for use as human vaccines. These analyses may include such factors as the relevance of host-vector interactions that may affect vaccine potency, overall safety of the vector, and the duration and minimal requirements for immunity to MBGV disease induced by this vector.

Two viral antigens demonstrated unambiguous potential as protective antigens in the guinea pig model: MBGV GP and MBGV NP. Another viral antigen, VP35, provided significant protection from death; however, most (5/6) animals vaccinated with VP35 exhibited viremias 7 days after infection. Consequently, VP35 was not considered a candidate for the initial examination of vaccine efficacy in nonhuman primates. While none of the other viral antigens showed significant promise as protective antigens in the guinea pig model, some were only weakly immunogenic, at least when delivered as VRPs. Thus, we have not formally excluded the possibility

that such antigens may prove protective under different circumstances, or in species other than guinea pigs.

As a more definitive test of efficacy, the two most promising guinea pig protective antigens from MBGV were used to inoculate nonhuman primates either alone or in combination. Using recombinant VEE replicons, MBGV GP was clearly shown to be protective. The observation that none of the animals developed overt illness or viremia was conclusive proof that this vaccine approach had protected animals from a substantial challenge dose of MBGV. However, there were some significant differences observed between guinea pigs and cynomolgus macaques. Most notable was the observation that two-thirds of the GP-vaccinated monkeys demonstrated rises in ELISA antibody titers following MBGV challenge, whereas there was apparently sterile immunity (i.e. no further increases in antibody titers) to viral challenge in guinea pigs given a 10-fold lower dose of the same vaccine. This may be attributable to the overall higher prechallenge ELISA antibody titers observed in guinea pigs when compared to those observed in the monkeys (Table 1 vs. Fig. 3).

The second antigen examined, MBGV NP, was less effective at protecting nonhuman primates compared to guinea pigs. All the monkeys inoculated with NP displayed signs of illness, with one animal dying in the same time frame as control animals. All animals were viremic, and viremia levels were predictive of outcome. As expected, the two animals that survived illness had large boosts in their ELISA antibody titers against MBGV when pre- and postchallenge sera were examined. Though not statistically significant in a group of only three animals, MBGV NP was

apparently able to provide a measure of protection from death, but not from disease in two monkeys. We surmise that the immune response to NP was sufficient to suppress replication of MBGV until augmented by additional host immune responses.

The monkeys that were vaccinated with both MBGV GP and NP demonstrated the same degree of protection as the animals vaccinated with GP alone. No viremias were observed at any time point, and two of three animals demonstrated postchallenge increases in ELISA antibody titers to MBGV. These results demonstrated that the NP replicon, equivocal by itself as a macaque vaccine, did not interfere with a GP-based vaccine when protective efficacy was used as a measurement.

For these studies, in the interest of expedient vaccine development, protection from viral disease was prioritized over the detailed study of immune mechanisms in two relatively difficult animal species for immunological studies, guinea pigs and cynomolgus macaques. It was already clear from studies done in guinea pigs that ELISA antibody titers to MBGV were not wholly predictive of clinical outcome, but rather one measure of immunogenicity of the vaccine candidate. However, it was also known that administration of polyclonal antisera or a neutralizing MAb could protect some guinea pigs from lethal challenge, indicating that antibodies can play a role in the protective response to MBGV (Hevey *et al.*, 1997, supra). As for immunity to virtually all viruses, T cell responses to MBGV are almost certainly important in their immunoregulatory and effector functions. Indeed, we observed protection in both guinea pigs (NP and VP35) and nonhuman primates (NP) with antigens for which the most logical protective mechanisms involve cellular immunity. However, it

also proved emphatically true in the most susceptible animals -- nonhuman primates -- that protective immunity was elicited by an antigen (GP) that theoretically favored a redundant protective response of both T cells and antibodies.

What is claimed is:

1. A recombinant DNA construct comprising:

(i) a vector, and

(ii) at least one of the MBGV virus DNA fragments encoding any one of GP, NP, VP40, VP35, VP30, VP24, and GPΔTM.

2. A recombinant DNA construct according to claim 1 wherein said vector is an expression vector.

3. A recombinant DNA construct according to claim 1 wherein said vector is a prokaryotic vector.

4. A recombinant DNA construct according to claim 1 wherein said vector is a eukaryotic vector.

5. The recombinant DNA construct of claim 1 wherein said vector is a VEE virus replicon vector.

6. The recombinant DNA construct according to claim 5 wherein said MBGV virus proteins are from strain Musoke.

7. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus GP.

8. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus NP.

9. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus VP40.

10. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus VP35.

11. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus VP30.

12. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus VP24.

13. The recombinant DNA construct according to claim 5 wherein said construct is pRep Mus GPΔTM.

14. Self replicating RNA produced from the construct of any of claim 7, 8, 9, 10, 11, 12, or 13.

15. Infectious alphavirus particles produced from packaging the self replicating RNA of claim 14.

16. A pharmaceutical composition comprising infectious alphavirus particles according to claim 15 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

17. A host cell transformed with a recombinant DNA construct according to claim 5.

18. A host cell according to claim 17 wherein said host cell is prokaryotic.

19. A host cell according to claim 17 wherein said host cell is eukaryotic.

20. A method for producing MBGV virus proteins comprising culturing the cells according to claim 18 under conditions such that said DNA fragment is expressed and said MBGV protein is produced.

21. A method for producing MBGV proteins comprising culturing the cells according to claim 19 under conditions such that said DNA fragment is expressed and said MBGV protein is produced.

22. A vaccine for MBGV comprising viral particles containing one or more replicon RNA encoding one or more MBGV proteins selected from the group consisting of GP, NP, VP24, VP30, VP35, VP40, and GPΔTM.

23. A pharmaceutical composition comprising the self replication RNA of claim 14 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

24. A pharmaceutical composition comprising one or more recombinant DNA constructs chosen from the group consisting of pRep Mus GP, pRep Mus GPΔTM, pRep Mus NP, pRep Mus VP40, pRep Mus VP35, pRep Mus VP30, pRep Mus VP24 in a pharmaceutically acceptable amount, in a pharmaceutically accpetable carrier/and or adjuvant.

FIG. 1

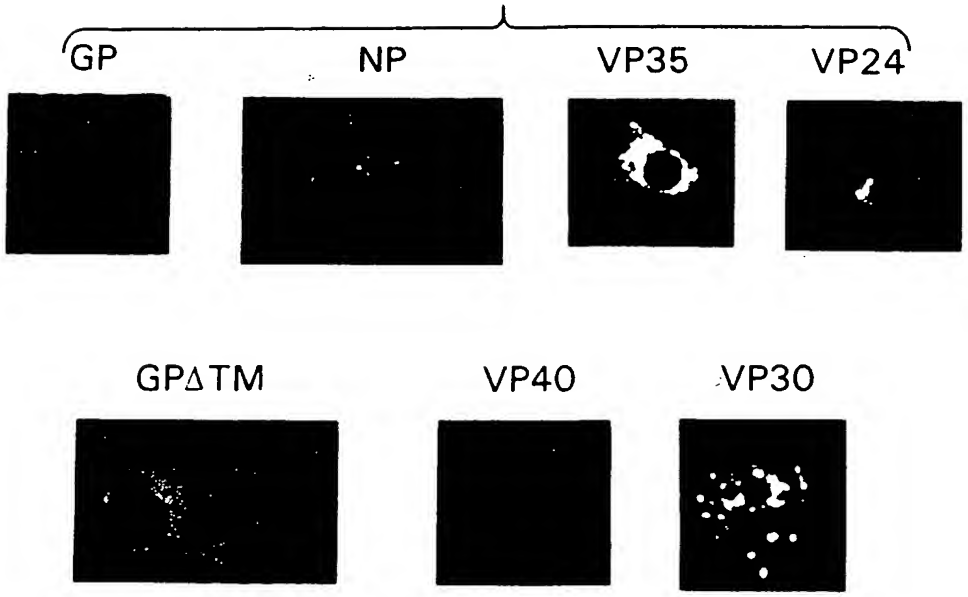


FIG. 2

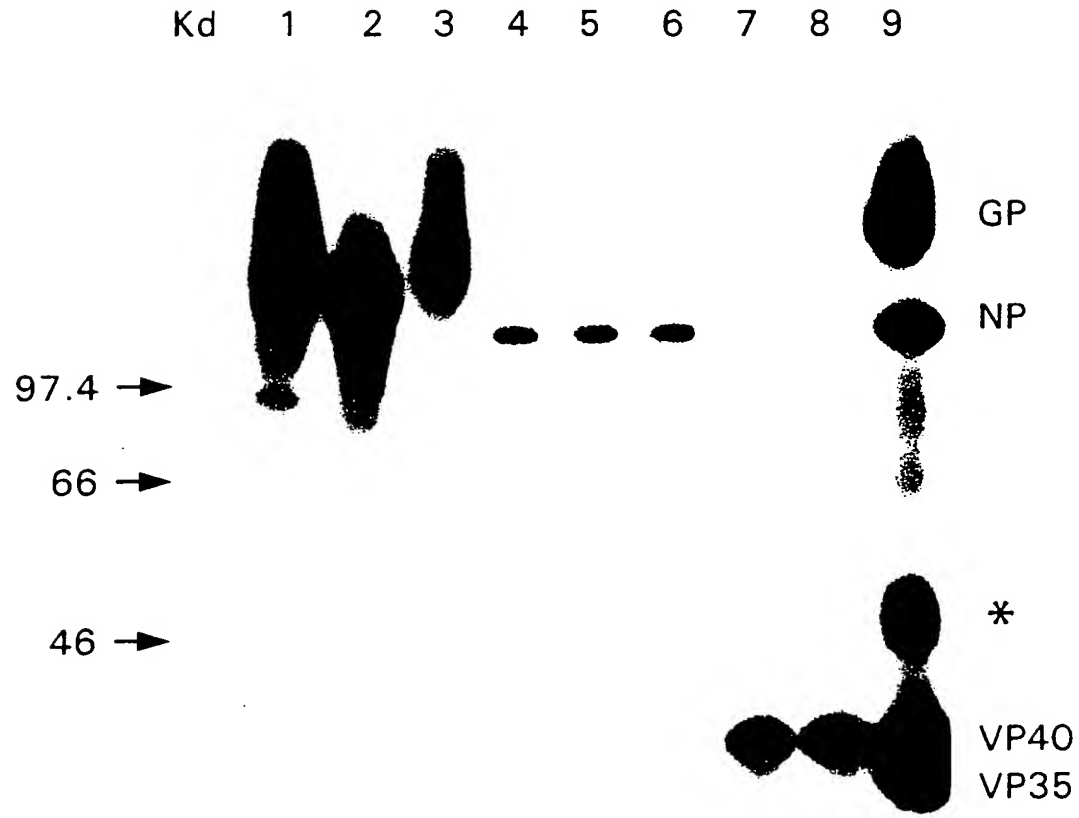
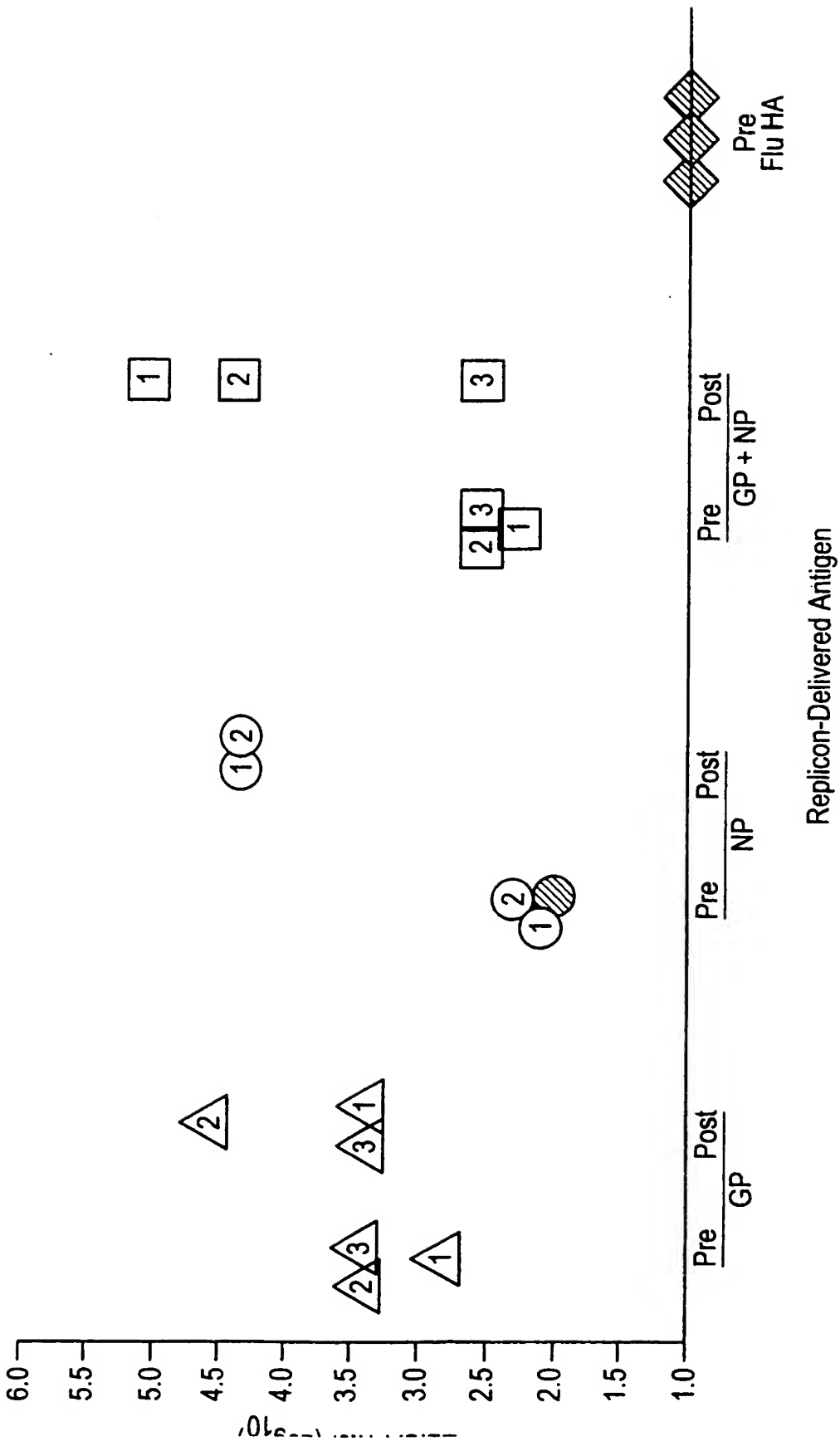


FIG. 3


6.0
5.5
5.0
4.5
4.0
3.5
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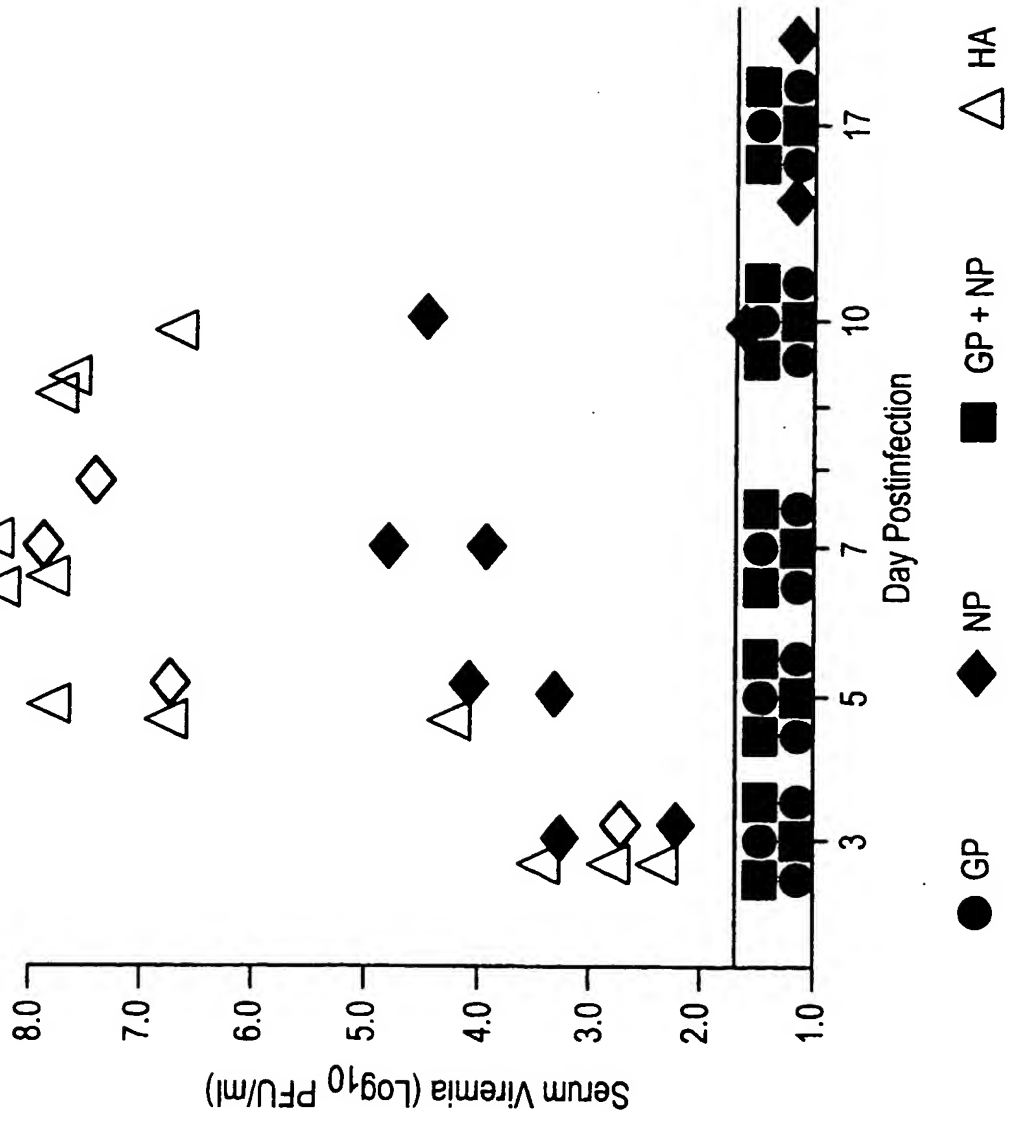
Pre
Post
GP
NP
GP + NP
Pre Flu HA
Replicon-Delivered Antigen

FIG. 4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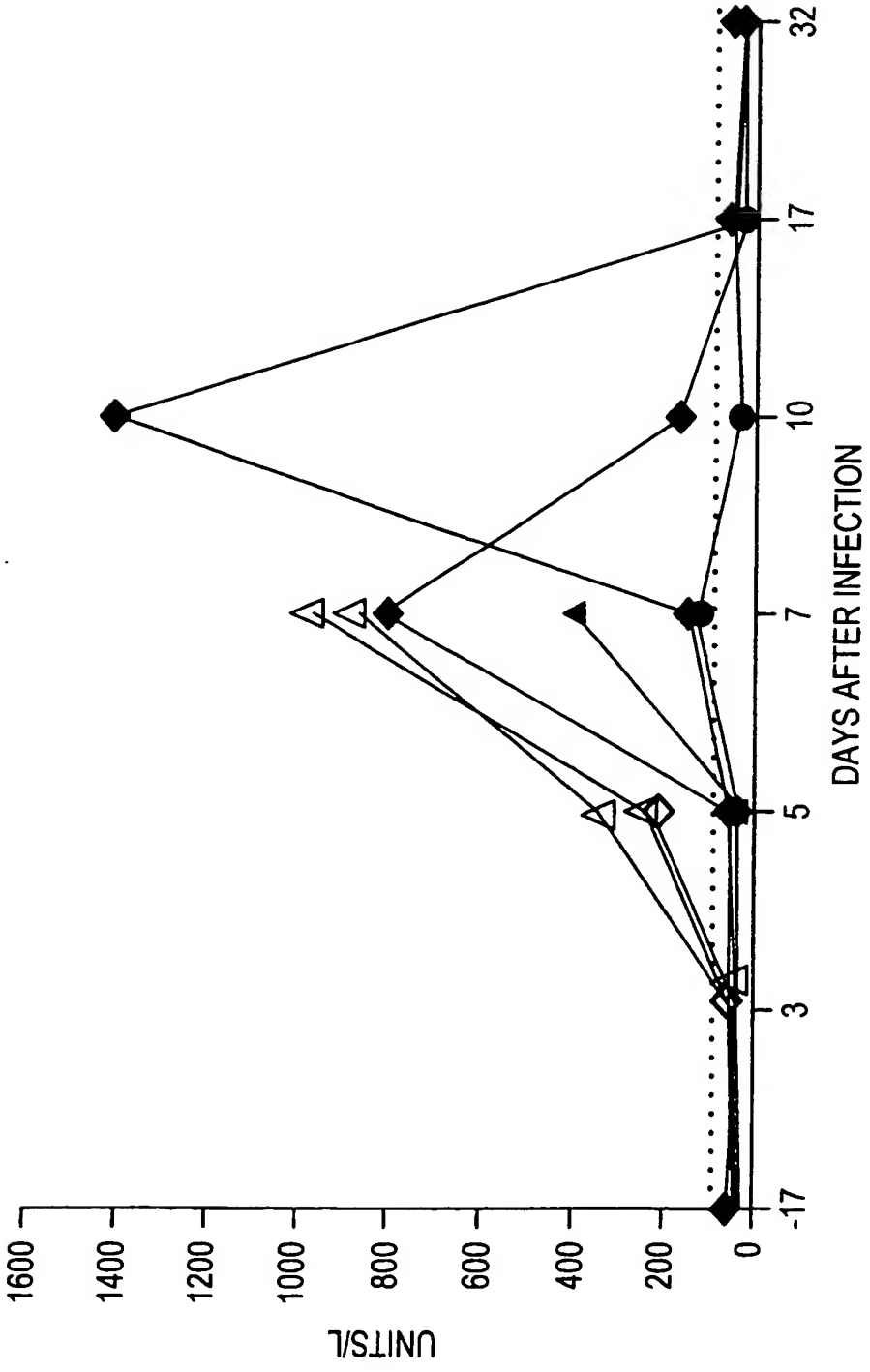
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
3
5
7
10
17
Day Postinfection
GP
NP
GP + NP
HA

FIG. 5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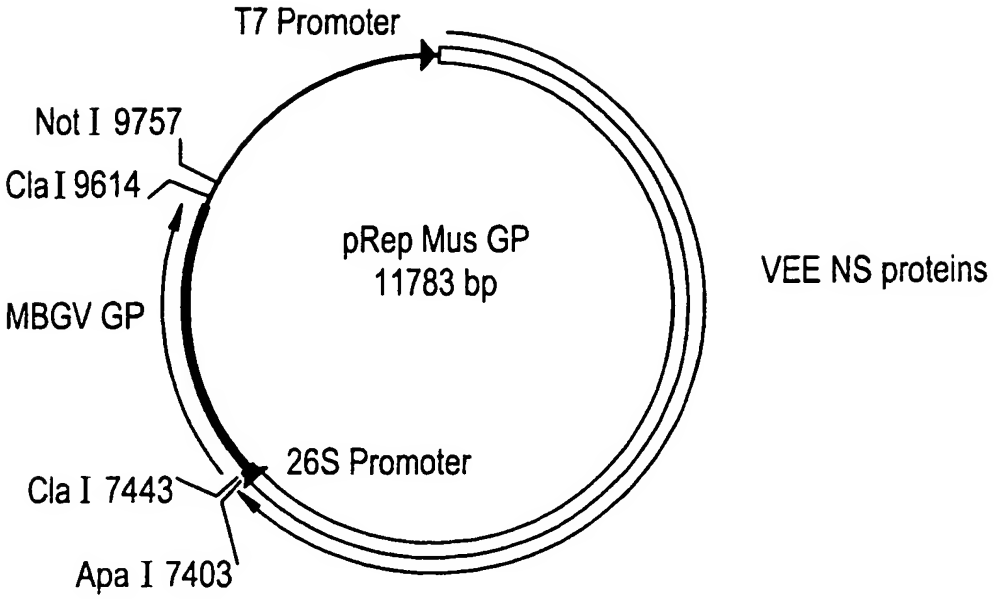
32
DAYS AFTER INFECTION

FIG. 6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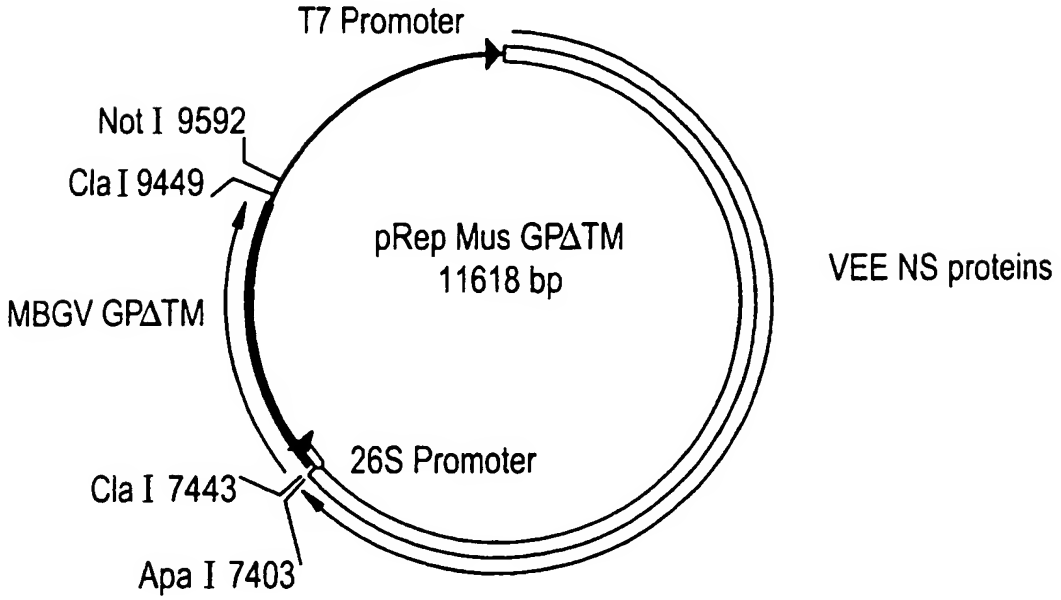
VEE NS proteins
MBGV GP
26S Promoter
Cla I 7443
Apa I 7403

FIG. 7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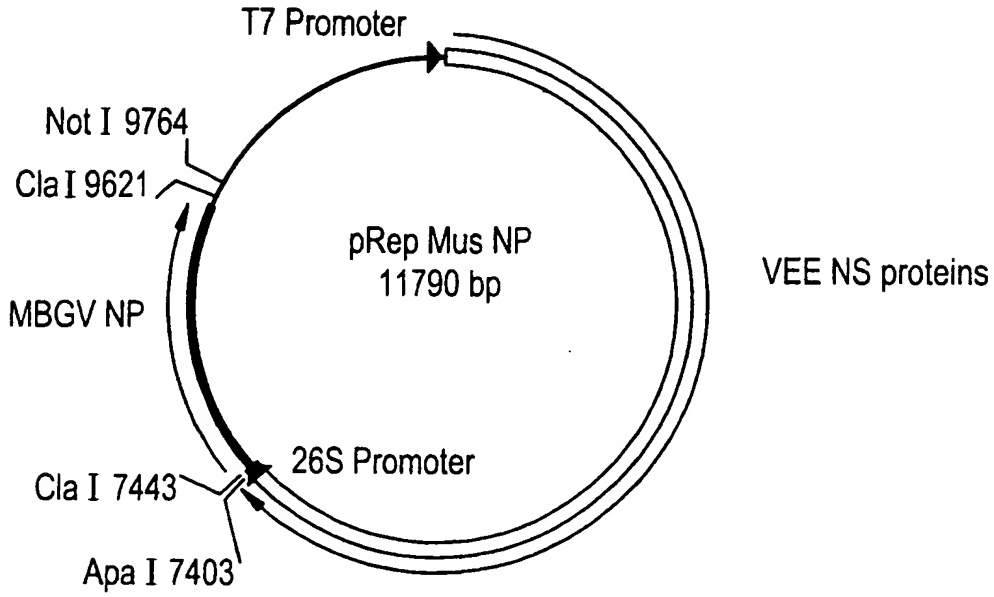
VEE NS proteins
MBGV GPΔTM
26S Promoter
Cla I 7443
Apa I 7403

FIG. 8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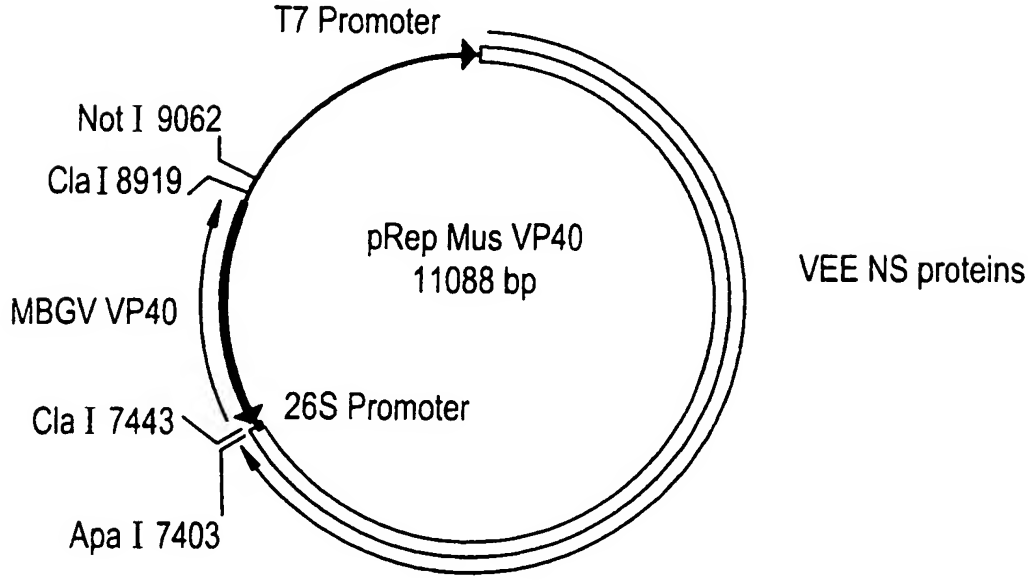
MBGV NP
26S Promoter
Cla I 7443
Apa I 7403

FIG. 9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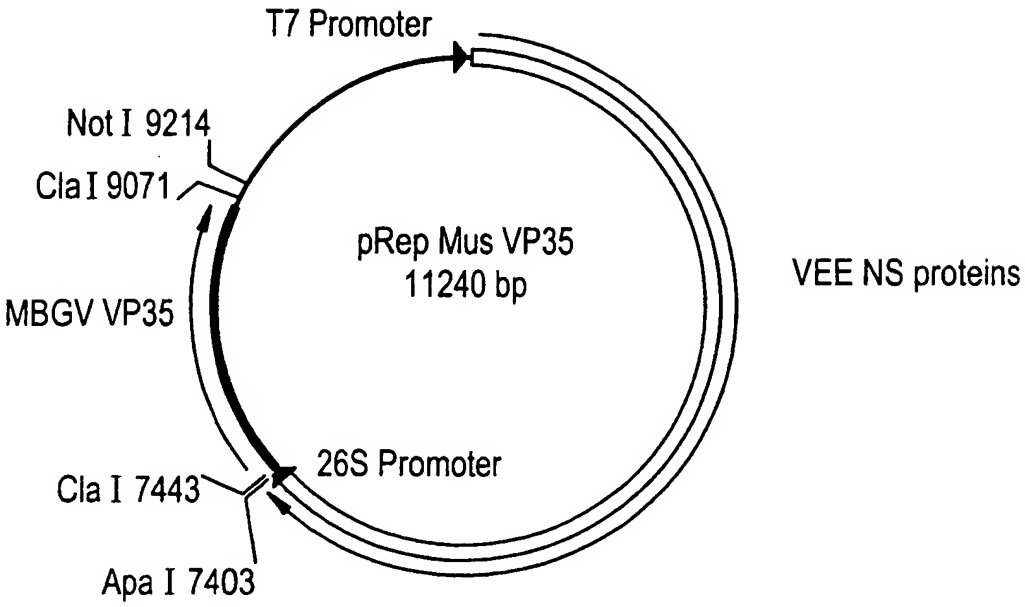
VEE NS proteins
MBGV VP40
26S Promoter
Cla I 7443
Apa I 7403

FIG. 10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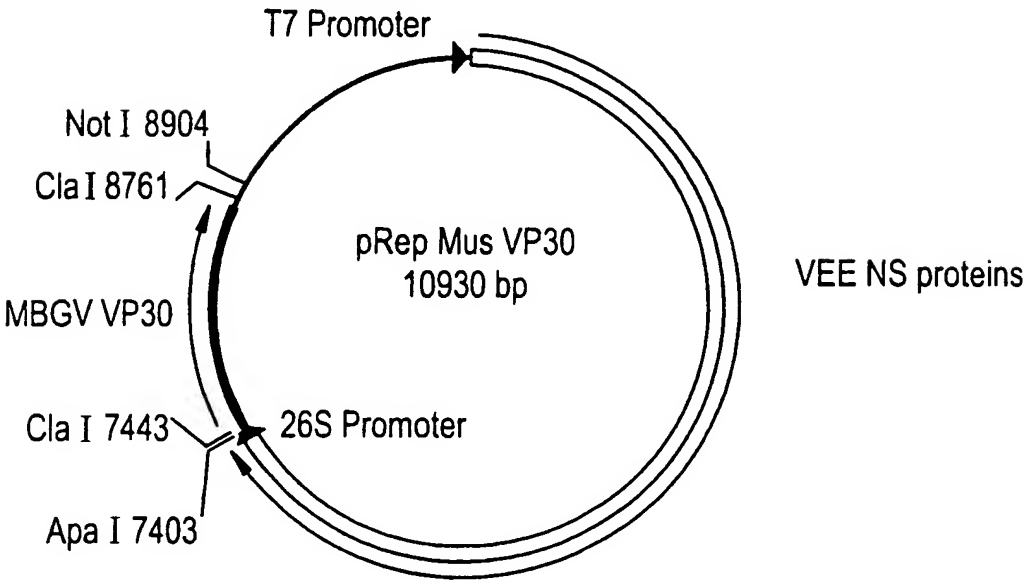
MBGV VP35
26S Promoter
Cla I 7443
Apa I 7403

FIG. 11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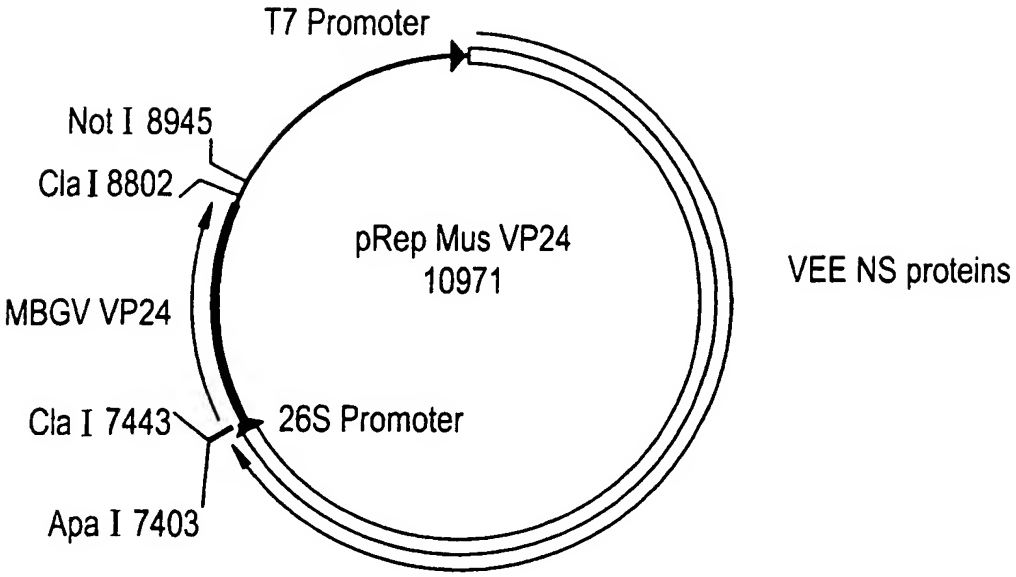
Cla I 7443
26S Promoter
Apa I 7403

FIG. 12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VEE NS proteins
MBGV VP24
Cla I 7443
26S Promoter
Apa I 7403

SEQUENCE LISTING

<110> United States Army Medical Research Institute of Infectious Diseases
Hevey, Michael C.
Negley, Diane L.
Pushko, Peter
Smith, Jonathan F.
Schmaljohn, Alan L.

<120> Marburg Virus Vaccines

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<210> 2

<211> 686

<212> PRT

<213> Marburg Virus

<220>

<400> 2

Met Lys Thr Thr Cys Phe Leu Ile Ser Leu Ile Leu Ile Gln Gly
1 5 10 15

Thr Lys Asn Leu Pro Ile Leu Glu Ile Ala Ser Asn Asn Gln Pro
20 25 30

Gln Asn Val Asp Ser Val Cys Ser Gly Thr Leu Gln Lys Thr Glu
35 40 45

Asp Val His Leu Met Gly Phe Thr Leu Ser Gly Gln Lys Val Ala
50 55 60

Asp Ser Pro Leu Glu Ala Ser Lys Arg Trp Ala Phe Arg Thr Gly
65 70 75

Val Pro Pro Lys Asn Val Glu Tyr Thr Glu Gly Glu Glu Ala Lys
80 85 90

Thr Cys Tyr Asn Ile Ser Val Thr Asp Pro Ser Gly Lys Ser Leu
95 100 105

Leu Leu Asp Pro Pro Thr Asn Ile Arg Asp Tyr Pro Lys Cys Lys
110 115 120

Thr Ile His His Ile Gln Gly Gln Asn Pro His Ala Gln Gly Ile
125 130 135

Ala Leu His Leu Trp Gly Ala Phe Phe Leu Tyr Asp Arg Ile Ala
140 145 150

Ser Thr Thr Met Tyr Arg Gly Lys Val Phe Thr Glu Gly Asn Ile
155 160 165

Ala Ala Met Ile Val Asn Lys Thr Val His Lys Met Ile Phe Ser
170 175 180

Arg Gln Gly Gln Gly Tyr Arg His Met Asn Leu Thr Ser Thr Asn
185 190 195

Lys Tyr Trp Thr Ser Ser Asn Gly Thr Gln Thr Asn Asp Thr Gly
200 205 210

Cys Phe Gly Ala Leu Gln Glu Tyr Asn Ser Thr Lys Asn Gln Thr
215 220 225

Cys Ala Pro Ser Lys Ile Pro Pro Pro Leu Pro Thr Ala Arg Pro
230 235 240

Glu Ile Lys Leu Thr Ser Thr Pro Thr Asp Ala Thr Lys Leu Asn
245 250 255

Thr Thr Asp Pro Ser Ser Asp Asp Glu Asp Leu Ala Thr Ser Gly
260 265 270

Ser Gly Ser Gly Glu Arg Glu Pro His Thr Thr Ser Asp Ala Val
275 280 285

Thr Lys Gln Gly Leu Ser Ser Thr Met Pro Pro Thr Pro Ser Pro
290 295 300

Gln Pro Ser Thr Pro Gln Gln Gly Gly Asn Asn Thr Asn His Ser
305 310 315

Gln Asp Ala Val Thr Glu Leu Asp Lys Asn Asn Thr Thr Ala Gln
320 325 330

Pro Ser Met Pro Pro His Asn Thr Thr Thr Ile Ser Thr Asn Asn
335 340 345

Thr Ser Lys His Asn Phe Ser Thr Leu Ser Ala Pro Leu Gln Asn
350 355 360

Thr Thr Asn Asp Asn Thr Gln Ser Thr Ile Thr Glu Asn Glu Gln
365 370 375

Thr Ser Ala Pro Ser Ile Thr Thr Leu Pro Pro Thr Gly Asn Pro
380 385 390

Thr Thr Ala Lys Ser Thr Ser Ser Lys Lys Gly Pro Ala Thr Thr
395 400 405

Ala Pro Asn Thr Thr Asn Glu His Phe Thr Ser Pro Pro Pro Thr
410 415 420

Pro Ser Ser Thr Ala Gln His Leu Val Tyr Phe Arg Arg Lys Arg
425 430 435

Ser Ile Leu Trp Arg Glu Gly Asp Met Phe Pro Phe Leu Asp Gly
440 445 450

Leu Ile Asn Ala Pro Ile Asp Phe Asp Pro Val Pro Asn Thr Lys

460 465 470

Thr Ile Phe Asp Glu Ser Ser Ser Ser Gly Ala Ser Ala Glu Glu
475 480 485

Asp Gln His Ala Ser Pro Asn Ile Ser Leu Thr Leu Ser Tyr Phe
490 495 500

Pro Asn Ile Asn Gly Asn Thr Ala Tyr Ser Gly Glu Asn Glu Asn
505 510 515

Asp Cys Asp Ala Glu Ley Arg Ile Trp Ser Val Gln Glu Asp Asp
520 525 530

Leu Ala Ala Gly Leu Ser Trp Ile Pro Phe Phe Gly Pro Gly Ile
535 540 545

Glu Gly Leu Tyr Thr Ala Val Leu Ile Lys Asn Gln Asn Asn Leu
550 555 560

Val Cys Arg Leu Arg Arg Leu Ala Asn Gln Thr Ala Lys Ser Leu
565 570 575

Glu Leu Leu Leu Arg Val Thr Thr Glu Glu Arg Thr Phe Ser Leu
580 585 590

Ile Asn Arg His Ala Ile Asp Phe Leu Leu Thr Arg Trp Gly Gly
595 600 605

Thr Cys Lys Val Leu Gly Pro Asp Cys Cys Ile Gly Ile Glu Asp
610 615 620

Leu Ser Lys Asn Ile Ser Glu Gln Ile Asp Gln Ile Lys Lys Asp
625 630 635

Glu Gln Lys Glu Gly Thr Gly Trp Gly Leu Gly Gly Lys Trp Trp
640 645 650

Thr Ser Asp Trp Gly Val Leu Thr Asn Leu Gly Ile Leu Leu Leu
655 660 665

Leu Ser Ile Ala Val Leu Ile Ala Leu Ser Cys Ile Cys Arg Ile
670 675 680

Phe Thr Lys Tyr Ile Gly
685

<210> 3

<211> 693

<212> PRT

<213> Marburg Virus

<220>

<400> 3

Met Asp Leu His Ser Leu Leu Glu Leu Gly Thr Pro Thr Ala Pro
1 5 10 15

His Val Arg Asn Lys Lys Val Ile Leu Phe Asp Thr Asn His Gln
20 25 30

Val Ser Ile Cys Asn Gln Ile Ile Asp Ala Ile Asn Ser Gly Ile
35 40 45

Asp Leu Gly Asp Leu Leu Glu Gly Gly Gly Leu Leu Thr Leu Cys
50 55 60

Val Glu His Tyr Tyr Asn Ser Asp Lys Asp Lys Phe Asn Thr Ser
65 70 75

Pro Val Ala Lys Tyr Leu Arg Asp Ala Gly Tyr Glu Phe Asp Val
80 85 90

Ile Lys Asn Ala Asp Ala Thr Arg Phe Leu Asp Val Ser Pro Asn
95 100 105

Glu Pro His Tyr Ser Pro Leu Ile Leu Ala Leu Lys Thr Leu Glu
110 115 120

Ser Thr Glu Ser Gln Arg Gly Arg Ile Gly Leu Phe Leu Ser Phe
125 130 135

Cys Ser Leu Phe Leu Pro Lys Leu Val Val Gly Asp Arg Ala Ser
140 145 150

Ile Glu Lys Ala Leu Arg Gln Val Thr Val His Gln Glu Gln Gly
155 160 165

Ile Val Thr Tyr Tyr Pro Asn His Trp Leu Thr Thr Gly His Met
170 175 180

Lys Val Ile Phe Gly Ile Leu Arg Ser Ser Phe Ile Leu Lys Phe
185 190 195

Val Leu Ile His Gln Gly Val Asn Leu Val Thr Gly His Asp Ala
200 205 210

Tyr Asp Ser Ile Ile Ser Asn Ser Val Gly Gln Thr Arg Phe Ser
215 220 225

Gly Leu Leu Ile Val Lys Thr Val Leu Glu Phe Ile Leu Gln Lys
230 235 240

Thr Asp Ser Gly Val Thr Leu His Pro Leu Val Arg Thr Ser Lys
245 250 255

Val Lys Asn Glu Val Ala Ser Phe Lys Gln Ala Leu Ser Asn Leu
260 265 270

Ala Arg His Gly Glu Tyr Ala Pro Phe Ala Arg Val Leu Asn Leu
275 280 285

Ser Gly Ile Asn Asn Leu Glu His Gly Leu Tyr Pro Gln Leu Ser
290 295 300

Ala Ile Ala Leu Gly Val Ala Thr Ala His Gly Ser Thr Leu Ala
305 310 315

Gly Val Asn Val Gly Glu Gln Tyr Glu Glu Leu Arg Glu Ala Ala
320 325 330

His Asp Ala Glu Val Lys Leu Gln Arg Arg His Glu His Gln Glu
335 340 345

Ile Gln Ala Ile Ala Glu Asp Asp Glu Glu Arg Lys Ile Leu Glu
350 355 360

Gln Phe His Leu Gln Lys Thr Glu Ile Thr His Ser Gln Thr Leu
365 370 375

Ala Val Leu Ser Gln Lys Arg Glu Lys Leu Ala Arg Leu Ala Ala
380 385 390

Glu Ile Glu Asn Asn Ile Val Glu Asp Gln Gly Phe Lys Gln Ser
395 400 405

Gln Asn Arg Val Ser Gln Ser Phe Leu Asn Asp Pro Thr Pro Val
410 415 420

Glu Val Thr Val Gln Ala Arg Pro Met Asn Arg Pro Thr Ala Leu
425 430 435

Pro Pro Pro Val Asp Asp Lys Ile Glu His Glu Ser Thr Glu Asp
440 445 450

Ser Ser Ser Ser Ser Ser Phe Val Asp Leu Asn Asp Pro Phe Ala
455 460 465

Leu Leu Asn Glu Asp Glu Asp Thr Leu Asp Asp Ser Val Met Ile
470 475 480

Pro Gly Thr Thr Ser Arg Glu Phe Gln Gly Ile Pro Glu Pro Pro
485 490 495

Arg Gln Ser Gln Asp Leu Asn Asn Ser Gln Gly Lys Gln Glu Asp
500 505 510

Glu Ser Thr Asn Arg Ile Lys Lys Gln Phe Leu Arg Tyr Gln Glu
515 520 525

Leu Pro Pro Val Gln Glu Asp Asp Glu Ser Glu Tyr Thr Thr Asp
530 535 540

Ser Gln Glu Ser Ile Asp Gln Pro Gly Ser Asp Asn Glu Gln Gly
545 550 555

Val Asp Leu Pro Pro Pro Pro Leu Tyr Ala Gln Glu Lys Arg Gln
560 565 570

Asp Pro Ile Gln His Pro Ala Ala Asn Pro Gln Asp Pro Phe Gly
575 580 585

Ser Ile Gly Asp Val Asn Gly Asp Ile Leu Glu Pro Ile Arg Ser
590 595 600

Pro Ser Ser Pro Ser Ala Pro Gln Glu Asp Thr Arg Met Arg Glu
605 610 615

Ala Tyr Glu Leu Ser Pro Asp Phe Thr Asn Asp Glu Asp Asn Gln
620 625 630

Gln Asn Trp Pro Gln Arg Val Val Thr Lys Lys Gly Arg Thr Phe
635 640 645

Leu Tyr Pro Asn Asp Leu Leu Gln Thr Asn Pro Pro Glu Ser Leu
650 655 660

Ile Thr Ala Leu Val Glu Glu Tyr Gln Asn Pro Val Ser Ala Lys
665 670 675

Glu Leu Gln Ala Asp Trp Pro Asp Met Ser Phe Asp Glu Gly Asp
680 685 690

Met Leu Arg

<210> 4

<211> 308

<212> PRT

<213> Marburg Virus

<220>

<400> 4

Met Ala Ser Ser Ser Asn Tyr Asn Thr Tyr Met Gln Tyr Leu Asn
1 5 10 15

Ser Pro Pro Tyr Ala Asp His Gly Ala Asn Gln Leu Ile Pro Ala

20 25 30

Asp Gln Leu Ser Asn Gln Gln Gly Ile Thr Pro Asn Tyr Val Gly
35 40 45

Asp Leu Asn Leu Asp Asp Gln Phe Lys Gly Asn Val Cys His Ala
50 55 60

Phe Thr Leu Glu Ala Ile Ile Asp Ile Ser Ala Tyr Asn Glu Arg
65 70 75

Thr Val Lys Gly Val Pro Ala Trp Leu Pro Leu Gly Ile Met Ser
80 85 90

Asn Phe Glu Tyr Pro Leu Ala His Thr Val Ala Ala Leu Leu Thr
95 100 105

Gly Ser Tyr Thr Ile Thr Gln Phe Thr His Asn Gly Gln Lys Phe
110 115 120

Val Arg Val Asn Arg Leu Gly Thr Gly Ile Pro Ala His Pro Leu
125 130 135

Arg Met Leu Arg Glu Gly Asn Gln Ala Phe Ile Gln Asn Met Val
140 145 150

Ile Pro Arg Asn Phe Ser Thr Asn Gln Phe Thr Tyr Asn Leu Thr
160 165 170

Asn Leu Val Leu Ser Val Gln Lys Leu Prp Asp Asp Ala Trp Arg
175 180 185

Pro Ser Lys Asp Lys Leu Ile Gly Asn Thr Met His Pro Ala Val
190 195 200

Ser Ile His Pro Asn Leu Pro Pro Ile Val Leu Pro Thr Val Lys
205 210 215

Lys Gln Ala Tyr Arg Gln His Lys Asn Pro Asn Asn Gly Pro Leu
220 225 230

Leu Ala Ile Ser Gly Ile Leu His Gln Leu Arg Val Glu Lys Val
235 240 245

Pro Glu Lys Thr Ser Leu Phe Arg Ile Ser Leu Pro Ala Asp Met
250 255 260

Phe Ser Val Lys Glu Gly Met Met Lys Lys Arg Gly Glu Asn Ser
265 270 275

Pro Val Val Tyr Phe Gln Ala Pro Glu Asn Phe Pro Leu Asn Gly
280 285 290

Phe Asn Asn Arg Gln Val Val Leu Ala Tyr Ala Asn Pro Thr Leu
295 300 305
Ser Ala Val

<210> 5

<211> 334

<212> PRT

<213> Marburg Virus

<220>

<400> 5

Met Trp Asp Ser Ser Tyr Met Gln Gln Val Ser Glu Gly Leu Met
1 5 10 15

Thr Gly Lys Val Pro Ile Asp Gln Val Phe Gly Ala Asn Pro Leu
20 25 30

Glu Lys Leu Tyr Lys Arg Arg Lys Pro Lys Gly Thr Val Gly Leu
35 40 45

Gln Cys Ser Pro Cys Leu Met Ser Lys Ala Thr Ser Thr Asp Asp
50 55 60

Ile Ile Trp Asp Gln Leu Ile Val Lys Arg Thr Leu Ala Asp Leu
65 70 75

Leu Ile Pro Ile Asn Arg Gln Ile Ser Asp Ile Gln Ser Thr Leu
80 85 90

Ser Glu Val Thr Thr Arg Val His Glu Ile Glu Arg Gln Leu His
100 105 110

Glu Ile Thr Pro Val Leu Lys Met Gly Arg Thr Leu Glu Ala Ile
115 120 125

Ser Lys Gly Met Ser Glu Met Leu Ala Lys Tyr Asp His Leu Val
130 135 140

Ile Ser Thr Gly Arg Thr Thr Ala Pro Ala Ala Ala Phe Asp Ala
145 150 155

Tyr Leu Asn Glu His Gly Val Pro Pro Pro Gln Pro Ala Ile Phe
160 165 170

Lys Asp Leu Gly Val Ala Gln Gln Ala Cys Ser Lys Gly Thr Met
175 180 185

Val Lys Asn Ala Thr Thr Asp Ala Ala Asp Lys Met Ser Lys Val
190 195 200

Leu Glu Leu Ser Glu Glu Thr Phe Ser Lys Pro Asn Leu Ser Ala
205 210 215

Lys Asp Leu Ala Leu Leu Leu Phe Thr His Leu Pro Gly Asn Asn
220 225 230

Thr Pro Phe His Ile Leu Ala Gln Val Leu Ser Lys Ile Ala Tyr
235 240 245

Lys Ser Gly Lys Ser Gly Ala Phe Leu Asp Ala Phe His Gln Ile
250 255 260

Leu Ser Glu Gly Glu Asn Ala Gln Ala Ala Leu Thr Arg Leu Ser
265 270 275

Arg Thr Phe Asp Ala Phe Leu Gly Val Val Pro Pro Val Ile Arg
280 280 290

Val Lys Asn Phe Gln Thr Val Pro Arg Pro Ser Gln Lys Ser Leu
295 300 305

Arg Ala Val Pro Pro Asn Pro Thr Ile Asp Lys Gly Trp Val Cys
310 315 320

Val Tyr Ser Ser Glu Gln Gly Glu Thr Arg Ala Leu Lys Ile
325 330

<210> 6

<211> 277

<212> PRT

<213> Marburg Virus

<220>

<400> 6

Met Gln Gln Pro Arg Gly Arg Ser Arg Thr Arg Asn His Gln Val
1 5 10 15

Thr Pro Thr Ile Tyr His Glu Thr Gln Leu Pro Ser Lys Pro His
20 25 30

Tyr Thr Asn Tyr His Pro Arg Ala Arg Ser Met Ser Ser Thr Arg
35 40 45

Ser Ser Ala Glu Ser Ser Pro Thr Asn His Ile Pro Arg Ala Arg
50 55 60

Pro Pro Ser Thr Phe Asn Leu Ser Lys Pro Pro Pro Pro Pro Lys
65 70 75

Asp Met Cys Arg Asn Met Lys Ile Gly Leu Pro Cys Ala Asp Pro
80 85 90

Thr Cys Asn Arg Asp His Asp Leu Asp Asn Leu Thr Asn Arg Glu
95 100 105

Leu Leu Leu Leu Met Ala Arg Lys Met Leu Pro Asn Thr Asp Lys
110 115 120

Thr Phe Arg Met Pro Gln Asp Cys Gly Ser Pro Ser Leu Ser Lys
125 130 135

Gly Leu Ser Lys Asp Lys Gln Glu Gln Thr Lys Asp Val Leu Thr
140 145 150

Leu Glu Asn Leu Gly His Ile Leu Ser Tyr Leu His Arg Ser Glu
155 160 165

Ile Gly Asn Trp Met Arg His Leu Arg Ala Ala Leu Ser Leu Thr
170 175 180

Cys Ala Gly Ile Arg Lys Thr Asn Arg Ser Leu Ile Asn Thr Met
185 190 195

Thr Glu Leu His Met Asn His Glu Asn Leu Pro Gln Asp Gln Asp
200 205 210

Gly Val Ile Lys Gln Thr Tyr Thr Gly Ile His Leu Asp Lys Gly
215 220 225

Gly Gln Phe Glu Ala Ala Leu Trp Gln Gly Trp Asp Lys Arg Ser
230 235 240

Ile Ser Leu Phe Val Gln Ala Ala Leu Tyr Val Met Asn Asn Ile
245 250 255

Pro Cys Glu Ser Ser Ile Ser Val Gln Ala Ser Tyr Glu Ser Phe
260 265 270

Tyr Ser Ser Ser Lys Ser Arg
275

<210> 7

<211> 253

<212> PRT

<213> Marburg Virus

<220>

<400> 7

Met Ala Glu Leu Ser Thr Arg Tyr Asn Leu Pro Ala Asn Val Thr
1 5 10 15

Glu Asn Ser Ile Asn Leu Asp Leu Asn Ser Thr Ala Arg Trp Ile
20 25 30

Lys Glu Pro Ser Val Gly Gly Trp Thr Val Lys Trp Gly Asn Phe
35 40 45

Val Phe His Ile Pro Asn Thr Gly Met Thr Leu Leu His His Leu
50 55 60

Lys Ser Asn Phe Val Val Pro Glu Trp Gln Gln Thr Arg Asn Leu
65 70 75

Phe Ser His Leu Phe Lys Asn Pro Lys Ser Thr Ile Ile Glu Pro
80 85 90

Phe Leu Ala Leu Arg Ile Leu Leu Gly Val Ala Leu Lys Asp Gln
95 100 105

Glu Leu Gln Gln Ser Leu Ile Pro Gly Phe Arg Ser Ile Val His
110 115 120

Met Leu Ser Glu Trp Leu Leu Leu Glu Val Thr Ser Ala Ile His
125 130 135

Ile Ser Pro Asn Leu Leu Gly Ile Tyr Leu Thr Ser Asp Met Phe
140 145 150

Lys Ile Leu Met Ala Gly Val Lys Asn Phe Phe Asn Lys Met Phe
155 160 165

Thr Leu His Val Val Asn Asp His Gly Lys Pro Ser Ser Ile Glu
170 175 180

Ile Lys Leu Thr Gly Gln Gln Ile Ile Ile Thr Arg Val Asn Met
185 190 195

Gly Phe Leu Val Glu Val Arg Arg Ile Asp Ile Glu Pro Cys Cys
200 205 210

Gly Glu Thr Val Leu Ser Glu Ser Val Val Phe Gly Leu Val Ala
215 220 225

Glu Ala Val Leu Arg Glu His Ser Gln Met Glu Lys Gly Gln Pro
230 235 240

Leu Asn Leu Thr Gly Tyr Met Asn Ser Lys Ile Ala Ile
245 250

INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 99/14174

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/40 C12N15/86 C12N7/01 C07K14/08 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 37616 A (UNIV NORTH CAROLINA ;US HEALTH (US); JOHNSTON ROBERT E (US); DAVIS) 28 November 1996 (1996-11-28)	1,2,4,5, 17,19,20
Y	page 8, line 29 -page 9, line 2; claims 1-37	7,14-16

X	C. WILL ET AL.: "Marburg virus gene encodes for the virion mmebrane protein, a type I transmembrane glycoprotein" J. VIROLOGY, vol. 67, no. 3, March 1993 (1993-03), pages 1203-1210, XP002128205 AM.SOC.MICROBIOL.,WASHINGTON,US cited in the application	1-4,6, 17-20
Y	the whole document	5,7, 14-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 January 2000	02. 05. 2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2	Authorized officer

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 99/14174

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A.A. BUKREYEV ET AL.: "The GP-protein of Marburg virus contains the region similar to the 'immunosuppressive domain' of oncogenic retrovirus P15E protein" FEBS LETTERS, vol. 323, no. 1,2, May 1993 (1993-05), pages 183-187, XP002128206 ELSEVIER, AMSTERDAM, NL	1-4,6, 17-20
Y	the whole document	5,7, 14-16

X	M. HEVEY ET AL.: "Antigenicity and vaccine potential of Marburg virus glycoprotein expressed by baculovirus recombinnats" VIROLOGY, vol. 239, 1997, pages 206-216, XP002128207 ACADEMIC PRESS, INC.,NEW YORK, US cited in the application	1-4,6, 17-20
Y	the whole document	5,7, 14-16

X	SANCHEZ A ET AL: "SEQUENCE ANALYSIS OF THE EBOLA VIRUS GENOME: ORGANIZATION, GENETIC ELEMENTS, AND COMPARISON WITH THE GENOME OF MARBURG VIRUS" VIRUS RESEARCH,NL,AMSTERDAM, vol. 29, no. 3, page 215-240 XP000198438 ISSN: 0168-1702	1-4,6, 17-20
Y	figure 8	5,7, 14-16

X	A. SANCHEZ ET AL.: "Variation in the glycoprotein and VP35 genes of Marburg virus strains" VIROLOGY, vol. 240, no. 1, 5 January 1998 (1998-01-05), pages 138-146, XP002128208 ACADEMIC PRESS, INC.,NEW YORK, US	1-4,6, 17-20
Y	the whole document	5,7, 14-16

Y	P. PUSHKO ET AL.: "Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: Expression of heterologous genes in vitro and imunization against heterologous pathogens in vivo" VIROLOGY, vol. 239, 1997, pages 389-401, XP002128209 ACADEMIC PRESS, INC.,NEW YORK, US the whole document	5,7, 14-16

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INTERNATIONAL SEARCH REPORT

Intern: ıl Application No
PCT/US 99/14174

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HEVEY M ET AL: "Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates." VIROLOGY, (1998 NOV 10) 251 (1) 28-37. JOURNAL CODE: XEA., XP002128210 the whole document	1-7, 14-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/14174

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-6,14-20 (partially); 7 (complete)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: (1-6,14-20)-partially; 7-complete

A recombinant DNA construct comprising (i) a vector, and (ii) at least one MBGV virus DNA fragment encoding GP; the recombinant DNA construct wherein said MBGV virus protein is from strain Musoke; said DNA construct wherein said vector is VEE; host cell transformed with said recombinant DNA construct; said recombinant DNA construct is pRep Mus GP; Self replicating RNA produced from said construct; Infectious alphavirus particles produced from packaging said self replicating RNA; a pharmaceutical composition comprising infectious alphavirus particles; a method for producing MBGV virus proteins;

2. Claims: (1-6,14-20)-partially; 8-complete

Idem as invention 1 but limited to NP respectively pRep Mus NP;

3. Claims: (1-6,14-20)-partially; 9-complete

Idem as invention 1 but limited to NVP40 respectively pRep Mus VP40;

4. Claims: (1-6,14-20)-partially; 10-complete

Idem as invention 1 but limited to VP35 respectively pRep Mus VP35;

5. Claims: (1-6,14-20)-partially; 11-complete

Idem as invention 1 but limited to VP30 respectively pRep Mus VP30;

6. Claims: (1-6,14-20)-partially; 12-complete

Idem as invention 1 but limited to VP24 respectively pRep Mus VP24;

7. Claims: (1-6,14-20)-partially; 13-complete

Idem as invention 1 but limited to GPdeltaTM respectively pRep Mus GPdeltaTM;

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna il Application No
PCT/US 99/14174

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9637616 A	28-11-1996	US 5792462 A	11-08-1998
		AU 5925696 A	11-12-1996
		CA 2220964 A	28-11-1996
		JP 11505719 T	25-05-1999